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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<p>(54) Title: ERYTHROPOIETIN MUTEINS WITH ENHANCED ACTIVITY</p> <p>(57) Abstract</p> <p>Novel modifications of erythropoietin (Epo) which improve the biological activity of the protein are provided. The modified Epo proteins (Epo muteins) may be used in the same manner as has been demonstrated for wild type Epo, except that relatively smaller doses are required due to the enhanced biological activity. Methods of using the Epo muteins for treatment of blood disorders are provided. A method of obtaining additional Epo muteins with enhanced biological activity is also provided.</p>			

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## Erythropoietin Muteins With Enhanced Activity

This invention was made with Government Support under grant nos. R01-HL42949 and R01-GM39900 sponsored by the National Institute of Health. The Government has certain rights in the invention.

### 5      *Cross-Reference to Related Applications*

This application is a continuation-in-part of U.S. Patent Application Serial No. 08/049,802 filed April 21, 1993 which is incorporated herein by reference.

### *Background*

10     This invention relates to erythropoietin in general and more particularly to modified erythropoietin proteins (erythropoietin muteins) having improved biological activity.

15     Erythropoietin (hereafter referred to as Epo) is a glycoprotein hormone which, in its mature form in humans, is 166 amino acids long with a molecular weight of 34 to 38 kD (Jacobs *et al.*, *Nature* 313: 806-809 (1985). Epo occurs in  $\alpha$ ,  $\beta$ , and asialo forms which differ slightly in their carbohydrate composition and biological activity (Dordal *et al.*, *Endocrinology* 116: 2293-2299 (1985)).

20     In terms of biological function, Epo has been recognized as the hematopoietic cytokine that regulates the process of red blood cell production, known as erythropoiesis. Erythropoiesis is a controlled physiological process which normally produces red blood cells in numbers which do not impede blood flow, but which are sufficient for oxygen transport.

25     The binding of Epo to its cognate receptor (D'Andrea, A.D., *et al.*, *Cell* 57:277-285 (1989)) on erythroid precursor cells in the bone marrow results in salvaging these cells from programmed cell death, known as

apoptosis (Koury, M.J., *et al.*, *Science* 248:378-381 (1990)), allowing them to proliferate and differentiate into circulating erythrocytes (red blood cells). Epo thus facilitates the formation of erythrocytes from erythroid precursor cells.

- 5        The level of Epo in circulation normally controls the rate of red blood cell production in the body. Typically, Epo is present at a low plasma concentration sufficient to maintain a steady state concentration of red blood cells by stimulating formation of just enough red blood cells to replace those lost through normal processes, such as aging. However, when an increase in  
10      the production of red blood cells is needed, the amount of Epo in circulation is increased. This red blood cell deficit may be caused, for example, by anemia, the general loss of blood through hemorrhage, over-exposure to radiation, prolonged unconsciousness, or exposure to high altitudes where oxygen intake is reduced.
- 15      In mammals, Epo is produced in the fetal liver and adult kidney, circulates in the bloodstream, and binds to receptors on committed progenitor cells in the bone marrow and other hematopoietic tissues, resulting in proliferation and terminal maturation of erythroid cells (Jelkmann, W., *Physiol. Rev.* 72:449 (1992)). The expression of Epo, both mRNA and  
20      protein, is markedly increased by hypoxia, owing to a 3' enhancer and to highly conserved elements in the promoter region (Semenza, G.L. *et al.*, *Proc. Natl. Acad. Sci. USA* 88:5680-1684 (1988); Beck, I. *et al.*, *J. Biol. Chem.* 266:15563-15566 (1991); Pugh, C.W. *et al.*, *Proc. Natl. Acad. Sci. USA* 88:10553-10557; Blanchard, K.L. *et al.*, *Mol. Cell Biol.* 12:5373-5385  
25      (1992)). This elegant servomechanism enables Epo to regulate the red cell mass of man and other animals.

Genes encoding Epo from a number of species have been studied. Thus far, the Epo genes of man (Jacobs, K. *et al.*, *Nature* 313:806-10 (1985); Lin, F.-K. *et al.*, *Proc. Natl. Acad. Sci. USA* 82:7580-7584 (1985)), a  
30      monkey (*Macaca fascicularis*) (Lin, F.-K. *et al.*, *Gene* 44:201-209 (1986)) and a rodent, the mouse (McDonald, J.D. *et al.*, *Mol. & Cell Biol.* 6:842-848

(1986); Shoemaker, C.B. *et al.*, *Mol. & Cell Biol.* 6:849-858 (1986)) have been cloned, sequenced, and expressed. There is a high degree of sequence homology in the coding region of the mature secreted proteins from these species.

5        In particular, the gene encoding human Epo has been cloned and used to produce recombinant Epo in cell culture. Recombinantly produced human Epo has general utility as a substitute for Epo isolated from natural sources. In addition, recombinant Epo has facilitated the production of large amounts of Epo free from deleterious substances associated with naturally derived Epo,  
10      which has allowed for greater availability and utility of this protein.

      Recombinantly produced Epo has proven especially useful for the treatment of patients suffering from impaired red blood cell production (Physicians Desk Reference (PDR), 1993 edition, pp 602-605). Recombinant Epo has proven effective in treating anemia associated with chronic renal  
15      failure and HIV-Infected individuals suffering from lowered endogenous Epo levels related to therapy with Zidovudine (AZT) (See PDR, 1993 edition, at page 602).

      Modifications of the Epo protein which would improve its utility as a tool for diagnosis or treatment of blood disorders are certainly desirable. In  
20      particular, modified forms of Epo exhibiting enhanced biological activity would be more effective and efficient than native Epo in the therapy setting when it is necessary to administer Epo to the patient, enabling administration less frequently and/or at a lower dose. Administration of reduced amounts of Epo would also presumably reduce the risk of adverse effects associated with  
25      Epo treatment, such as hypertension, seizures, headaches, etc. (See PDR, 1993 edition, at pp. 603-604).

      Unfortunately, available information regarding the structure of the Epo enzyme and its function does not permit the accurate prediction of modifications which would result in such improvements. In fact, the highly  
30      conserved nature of the amino acid sequence of Epo indicates that this protein is, for the most part, resistant to change and that most modifications would be

expected to have deleterious effects on Epo and its activity (See U.S. Patent No. 4,835,260 by Shoemaker).

Some speculation does, however, exist regarding the possibility of creating variants of Epo with altered characteristics that would be preferable to the wild type protein for particular purposes. A number of modifications of Epo have been proposed, including deletions, additions, and substitution of existing amino acids. These modifications have been proposed as a means of achieving a variety of effects, including separation of various Epo functions on individual protein fragments, improving the efficacy of recombinant Epo production, improving *in vivo* stability, etc. Of particular relevance to the present invention are those modifications proposed to improve the biological activity (i.e. ability to stimulate red blood cell production) of Epo.

U.S. Patent No. 4,703,008 by Lin, F-K. (hereinafter referred to as "the '008 patent") speculates about a wide variety of modifications of Epo, including addition, deletion, and substitution analogs of Epo. The '008 patent does not indicate that any of the suggested modifications would increase biological activity *per se*, although it is stated that deletion of glycosylation sites might increase the activity of Epo produced in yeast (See the '008 patent at column 37, lines 25-28). Also, the '008 patent speculates that Epo analogs which have one or more tyrosine residues replaced with phenylalanine may exhibit an increased or decreased receptor binding affinity.

Australian Patent Application No. AU-A-59145/90 by Fibi, M *et al.* (herein referred to as "Fibi") also discusses a number of modified Epo proteins (Epo muteins). Fibi generally speculates about the alteration of amino acids 10-55, 70-85, and 130-166 of Epo. In particular, additions of positively charged basic amino acids in the carboxyl terminal region are purported to increase the biological activity of Epo.

U.S. Patent No. 4,835,260 by Shoemaker, C. B. discusses modified Epo proteins with amino acid substitutions of the methionine at position 54 and asparagine at position 38. Such Epo muteins are thought to have improved

stability but are not proposed to exhibit any increase in biological activity relative to wild type Epo.

Beyond the few proposed modifications that have been speculated to enhance the biological activity of Epo, the art provides no further guidance regarding how Epo can be modified to increase its biological activity. This lack of guidance indicates that, based upon the present state of knowledge, other modifications of Epo which may increase biological activity cannot be predicted and may not even exist.

The present invention represents the inventors' achievement in overcoming the lack of guidance and unpredictability regarding modifications of the Epo protein which increase its biological activity.

### *Summary Of The Invention*

It is therefore an object of the present invention to provide Epo muteins with enhanced biological activity.

It is a further object of the present invention to provide recombinant DNA encoding Epo muteins and methods of producing biologically active Epo muteins in a host cell culture using such recombinant DNA.

It is a further object of the present invention to provide methods of treating blood disorders involving abnormally low red blood cell populations using Epo muteins at dosages lower than that required for unmodified Epo to achieve the same affect.

It is a further object of the present invention to provide a method of obtaining additional Epo muteins with enhanced biological activity.

### Brief Description Of The Drawings

**Figure 1.** Oligonucleotide Primer Sequences. Oligonucleotide primers corresponding to Epo DNA sequences are shown. The localization of the primer coincides with previously published nucleotide sequences for the 5 human (Lin, F.-K. *et al.*, *Proc. Natl. Acad. Sci. USA* 82:7580-7584 (1985)) and murine (McDonald, J.D. *et al.*, *Mol. & Cell Biol.* 6:842-848 (1986)) Epo genes. EX2R and EX5 are not completely conserved between human and mouse (respectively 93% and 95% identity). An equal amount of each possible nucleotide was incorporated during the corresponding cycles of those 10 primer syntheses. EX2R and NCO1 are reverse primers and their sequences represent the antisense DNA strand.

**Figure 2.** PCR Strategy used for the cloning of mammalian cDNAs containing the complete coding sequence of the mature Erythropoietin Protein. Genomic amplification and sequencing of exonic 15 fragments, localized upstream and downstream from the nucleotide portion coding for the mature protein, allowed the design of species specific primers (SP). Utilization of those SP primers and/or of, sequences that are 100% conserved between man and mouse 5' ATG and 3' NCO1 primers (Figure 1) on cDNA templates prepared from kidney of uninduced or hypoxia-induced 20 animals, allows the amplification of a large variety of mammalian Epo clones. Sense (→) and antisense (←) primers are represented by the arrows. Dashed boxes correspond to the coding part (propeptide and mature protein) of the five Epo exons. Gray boxes represent the 5' and 3' untranslated regions.

**Figure 3.** Comparison of IV1/EX2R sequences from various 25 mammals. The numbering corresponds to the published human genomic sequence (Lin, F.-K. *et al.*, *Proc. Natl. Acad. Sci. USA* 82:7580-7584 (1985)). The reported human sequence was obtained from the amplification of purified genomic DNA from Hep3B cells and agreed with the sequence 30 previously reported. The mouse sequence is from McDonald, J.D. *et al.*, *Mol. & Cell Biol.* 6:842-848 (1986). The horse sequence was obtained from

kidney-extracted genomic DNA. All the other sequences were established from PCR amplification of genomic DNAs purified from several mammalian renal-derived cell lines. The consensus sequence indicates the positions where a unique nucleotide was found in all the reported species. The boundary 5 between intron 1 and exon 2 is represented by the ascending arrows. The locations of the two PCR primers, IV1 and EX2R are shown by the dashed arrows.

**Figure 4.** Alignment of the nucleotide sequences of mammalian Epo cDNAs. The mouse sequence corresponds to the one previously reported 10 (McDonald, J.D. et al., *Mol. & Cell Biol.* 6:842-848 (1986)). PCR-produced human sequence was obtained from hypoxia-induced Hep3B cell line and is in total agreement with the previously published sequence (Jacobs, K. et al., *Nature* 313:806-810 (1985); Lin, F.-K. et al., *Proc. Natl. Acad. Sci. USA* 82:7580-7584 (1985)). Monkey (*Macaca mulatta*), rat, sheep, pig and cat 15 were amplified from kidney-purified cDNAs. Amplifications of the human, monkey, rat and sheep were realized using the ATG (residues 1 to 20) and NCO1 (residues 723-742) primers. The line under residues 41 to 56 represents the specific SP1 primer used for the cloning of the pig and cat sequences. For these two mammals, 5' sequences for SP1 are derived from 20 the data presented in Figure 3. Arrows indicate the start and the end of the coding sequence (propeptide and mature protein).

**Figure 5.** Predicted Amino Acid Sequences of Epo Propeptide. The amino acid sequences are derived from data obtained from both genomic 25 IV1/EX2R and cDNA amplifications. The numbering corresponds to the human sequence. Ala 1 is the first amino acid of the human mature protein. The ascending arrows show the site of the cleavage by the signal peptidase, as determined for the human and cynomolgus monkey proteins.

**Figure 6.** Alignment of the Primary Structures of mature 30 mammalian Epo Proteins. The human, *Macaca fascicularis* and mouse amino acid sequences were previously reported (Lin, F.-K. et al., *Proc. Natl. Acad. Sci. USA* 82:7580-7584 (1985); Lin, F.-K. et al., *Gene* 44:201-209

(1986); McDonald, J.D. *et al.*, *Mol. & Cell Biol.* 6:842-848 (1986)). The residue numbers correspond to the 166 aa of the mature human protein. Plain boxes indicate the positions of the predicted four  $\alpha$ -helices in the human sequence (See Example II). Dashed boxes show the N- and O-glycosylation sites. Limits between exons are indicated by the vertical lines.

5        *Figure 6A.* Schematic representation of the mammal erythropoietin. The invariant amino acids among the eight sequences shown in Figure 6 are represented by the black boxes. The localization of each of the four  $\alpha$ -helices is underlined. The three glycosylation sites are shown as  
10      diamonds. The main disulfide bridge is indicated by the heavy line connecting noncontiguous sequences. The small arrows under the sequence indicate the short SH-bridge, missing in the rodents.

15        *Figures 7-7B.* Phylogenetic lineages derived from analyses of mammalian Epo cDNA sequences encoding the full length mature proteins from seven species.

20        *Figure 7.* Strength of groups in the maximum parsimony tree found on examining all 945 unrooted trees formed by seven terminal taxa. This tree of lowest length required 374 base substitutions. Each link between ancestral nodes has a circled number; this strength of grouping number is the minimum number of substitutions that must be added to the length of the maximum parsimony tree to find a tree that breaks down the barrier (moves one or more sequences) between the two groups separated by the interior link.

25        *Figure 7A.* The phylogenetic tree derived from the maximum parsimony reconstruction. On the basis of other molecular evidence involving comparative amino acid sequence data from monotremes, marsupials, and many eutherian species (Czelusniak, J. *et al.*, *Meth. Enzymol.* 183:601-615 (1990); Czelusniak, J. *et al.*, In "Current Mammalogy", Volume 2, 3rd ed., H. H. Ganoways, pp. 545-572, Plenum Publ. Corp.  
30      (1990)) the root of this Epo phylogenetic tree is placed on the interior link separating the artiodactyl group from the primate, rodent, cat group. The numbers on the internodal links represent the numbers of base pairs by which

the nodal ancestral and descendant sequences differ. MYA = millions of years ago, as inferred from paleontological views of eutherian phylogeny.

Figure 7B. Parsimony reconstruction on that portion of the cDNA sequences that are codons for amino acids. The numbers shown as a fraction on each link are, in the numerator, the number of amino acid changing base replacements and, in the denominator, the number of silent base replacements. The computer algorithm that carried out this calculation is described in Czelusniak, J. et al., *Nature* 298:297-300 (1982) (see the legend for Figure 2).

Figures 8-8A. Phylogenetic lineages derived from analyses of the mammalian Epo intron 1-exon 2 sequences.

Figure 8. Strength of groups in the maximum parsimony tree found on examining all 135,135 trees formed by nine terminal taxa. As the two feloid sequences (those of cat and lion) are nearly identical, they were treated as a single taxon in this strength of grouping analysis. The maximum parsimony tree for the segment (about 285 bp) of intron 1 and exon 2 from ten species required 361 base substitutions. The circled numbers are the strength of grouping numbers (as defined in the Figure 7 legend).

Figure 8A. The near most parsimonious tree that groups dog with feloids. This tree required 365 base substitutions. The numbers on links represent numbers of base pairs by which the nodal ancestral and descendant sequences differ.

Figures 9-9B. Model of the Three-Dimensional Structure of Erythropoietin.

Figure 9. Ribbon diagram of Epo tertiary structure. The four  $\alpha$  helices are labeled A to D; loops between helices are appropriately named. Disulfide bridges are shown and N- and O-glycosylation sites are indicated respectively by dark and dotted segments.

Figure 9A. Schematic representation of Epo's primary structure depicting predicted up-up-down-down orientation of the four antiparallel  $\alpha$

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helices (boxes with arrowhead). This folding pattern is strongly suggested by the large size of the two interconnecting loops AB and CD. The limits of each helix were drawn according to Table II of Example I. A predicted short region of  $\beta$ -sheet is delineated by the dashed rectangle. The N-glycosylation sites are represented by the dotted diamonds, the O-glycosylation site by the dashed oval. The locations of the two disulfide bridges are shown as solid lines.

Figure 9B. *Cross-section of the Epo molecule at the level of the four  $\alpha$  helices.* The helical wheel projections are viewed from the NH<sub>2</sub>-end of each helix. The hydrophobic residues, localized inside the globular structure, are indicated by filled circles. The charged and neutral residues (open and gray circles respectively) are exposed at the surface of the molecule.

Figure 10. Immunoprecipitations of wild type Epo and the  $\Delta$ 140-144 mutein. Cos7 cells were transfected with pSG5, pSG5-Epo/wt or pSG5-Epo/ $\Delta$ 140-144. After three days, the cells were metabolically labeled with [<sup>35</sup>S]-methionine and [<sup>35</sup>S]-cysteine. Immunoprecipitations of cellular extracts and supernatants were performed with our polyclonal antibody, raised in rabbit against the native human Epo. The immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis. Lanes 2 to 4 correspond to cellular extracts, and lanes 5 to 7 correspond to culture supernatants, from Cos7 transformed with the following: plasmid without insert (lanes 2 and 5), wild type Epo (lanes 3 and 6), and  $\Delta$ 140-144 (lanes 4 and 7). Lane 1 represents the protein molecular weight standard. The two arrows show the normal secretion of the wild type Epo (35-37 kD) and the cytoplasmic retention of the mutein  $\Delta$ 140=144 (~28 kD).

*Figures 11-11B.* Interconnecting Loop AB.

*Figure 11.* Schematic representation of the loop AB showing the localization of muteins with various deletions and amino acid replacements. The dashed arrows point to the positions of the serine substitutions (in Δ48-52). The two N-glycosylation sites are represented by gray diamonds. The small Cys29=Cys33 disulfide bridge is indicated.

*Figure 11A.* Amount and biological activities of secreted muteins. The upper bar graphs show the relative secretion of wild type and loop AB muteins as determined by radioimmunoassay. The lowest bar graphs display the calculated specific activity (ratio bioassay/RIA) for each mutein, in comparison with the value obtained for the wild type Epo (ratio = 100%).

*Figure 11B.* HCD57 cell proliferation as a function of increasing concentration of wild type and serine-substituted Epo muteins. HCD57 cells ( $10^4$  per ml) were cultured for three days in a 96 well microtiter plate with media containing increasing concentrations of secreted proteins (mU=milli-units of Epo, a relative mass measurement). The line graphs show the cellular growth as measured by  $^3\text{H}$ -thymidine uptake for cells cultured with wild type Epo (O), Epo mutein F48S ( $\diamond$ ), Epo mutein Y49S (X), Epo mutein A50S ( $\blacklozenge$ ), Epo mutein W51S ( $\square$ ), and Epo mutein K52S ( $\triangle$ ). The number of viable cells was also measured with the MTT colorimetric assay and gave similar curves. Proliferation experiments using the human UT-7/Epo cell line (Komatsu, N., et al., *Cancer Res.* 51:341-348 (1991)) and the Krystal assay (Krystal, G., *Exp. Hematol.* 11:649-60 (1983)) produced identical results.

*Figures 12-12A.* Interconnecting Loop CD.

*Figure 12.* Schematic representation of the loop CD showing the location of three deletion muteins: Δ105-109, Δ111-119, Δ122-126, and the insertion of seven residues after Lys116 (myc epitope). The O-glycosylation site is indicated by the dashed oval.

*Figure 12A.* Secretion and biological activities of the muteins located in loop CD. The two bar graphs were created as described in

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Figure 11A. The two mutants  $\Delta$ 111-119 and 116/myc were normally secreted and had full biological activities.

*Figures 13-13A. In Vitro Translation of the Epo Wild Type.*

*Figure 13. Analysis of the  $^{35}$ S-labeled translation products by*

- 5 **SDS-PAGE.** One-step transcription/translation reactions were performed in the SP6-TnT rabbit reticulocyte lysate system. 1/30 of each reaction was resolved on a 15% polyacrylamide gel. Lane 1-low Mr standard from Amersham; lane 2-*in vitro* reaction without added plasmid; lanes 3 and 4 are translation products obtained after incubation of 1  $\mu$ g of circular p64T-Epo,  
10 in the presence or absence of canine pancreatic microsomal membranes, respectively.

- 15 *Figure 13A. Binding of the in vitro translated Epo wild type onto Epo Receptor-GTS-agarose beads.  $6 \times 10^3$  cpm (counts per minute) of purified  $^{35}$ S-labeled erythropoietin products, processed with microsomes (+) or not (-) were incubated in the presence of the extra-cytoplasmic domain of the Epo receptor (ERE<sub>x</sub>), following the protocol described by Harris, K.W., et al., J. Biol. Chem. 267:15205-15209 (1992). Identical binding demonstrated that the conservation of the propeptide did not impair the hormone/receptor interaction.*

20 *Figures 14-14A. COOH-end of Epo.*

- Figure 14. Schematic representation of the analyzed muteins, corresponding to the deletion of the four last amino acids  $\Delta$ 163-166 and the replacements of the residues 162 to 166 by a KDEL or poly (His) sequences.*

- 25 *Figure 14A. Relative secretion of these muteins. The bioactivities in the supernatants (Krystal, G., Exp. Hematol. 11:649-60 (1983)) and the cell extracts (Komatsu, N., et al., Cancer Res. 51:341-348 (1991)) of transformed Cos7 cells were measured by *in vitro* proliferation assay using HCD57. More KDEL mutant remained in the cytosol of the Cos7, when compared with the wild type Epo and  $\Delta$ 163-166 or poly (His) muteins. However, all the analyzed muteins had the same specific activity as that of the wild type.*

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*Figure 15.* Bacterial Expression of Wild Type Epo.

*Figure 15 Panel A.* Diagram of the fusion protein. An NH<sub>2</sub>-terminal 22 amino acid long peptide, containing a 10 histidine stretch, was fused to the mature erythropoietin sequence. Factor Xa cleavage allowed the recovery of the mature Epo with only two extra residues at its amino terminus.

*Figure 15 Panel B.* IPTG induction of the fusion protein. Transformed *E. coli* BL21 (DE3) cultures ( $OD_{600}=0.6$ ) were grown in presence of 1 mM IPTG. Aliquots were collected at 0 (lane 2), 1 (lane 3), 2 (lane 4) and 3 hours (lane 5) and analyzed on a 15% SDS-polyacrylamide gel, stained with Coomassie Brilliant Blue. High level production of the fusion protein was rapidly obtained. Lane 6 corresponds to an aliquot from transformed bacteria grown for 3 hours in a medium without IPTG. Lane 1 is a low molecular weight standard.

*Figure 15 Panel C.* Purification of the fusion protein. After 3 hours of IPTG induction, the produced (His)<sub>10</sub>-Epo was solubilized in 6 M guanidine-HCl and purified on a nickel affinity resin by increasing imidazole concentrations following the pET-His system protocol (Novagen). Samples of the column eluants were analyzed by SDS-PAGE and stained with Coomassie Brilliant Blue. Lane 1-elution by 20 mM imidazole; lane 2-elution by 100 mM imidazole, releasing the fusion protein; lane 3-chelation of the nickel by a 100 mM EDTA wash; lane 4-molecular weight standard.

*Figure 15 Panel D.* Detection of the *E. coli* recombinant Epo on a Western blot. Solubilized proteins were separated on a 15% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane and probed with a 1/2000 dilution of our native wild type polyclonal antibody, as described under Materials and Methods. Lane 1-analysis after oxidative reduction; lane 2-after dialysis against the factor Xa buffer; and lane 3-after factor Xa cleavage.

*Figure 16.* Relationship Between Production of Muteins and Proposed Secondary Structure. This bar graph shows the amount of secreted proteins in the supernatants of transiently expressed Epo mutants, as

detected by radioimmunoassay. The muteins were aligned over a schematic representation of the native Epo molecule. Each deletion is shown as a stippled bar, the width of which is proportional to the number of residues deleted. The four  $\alpha$  helices are represented by the black rectangles. The two disulfide bridges are indicated. These mutagenesis results are in good agreement with our proposed four  $\alpha$ -helical model of Epo.

**Figure 17. Biological Activity of Helix A Muteins.** The top panel shows a helical wheel projection of Epo helix A with arrows indicating the positions where modifications were made to create muteins. The lower panel is a dose response curve representing the biological activity of wild type Epo (■) and helix A muteins E13A (◆), R14A (□), E18A (◇), K20A (×), and E21A (□) over a range of dosages (X-axis; mU=milli-units of Epo, a relative mass measurement) as determined by the relative incorporation of [<sup>3</sup>H]-thymidine measured in counts per minute (cpm) (Y-axis) into Epo dependent HCD57 cells.

**Figure 18. Biological Activity of Helix D Muteins.** The top panel shows a helical wheel projection of Epo helix D with arrows indicating the positions where modifications were made to create muteins. The lower panel is a dose response curve representing the biological activity over a range of dosages (X-axis) of wild type Epo (■) and helix D muteins D136A (◆), R139A (□), K140A (◇) and 143A (x) as determined by the relative incorporation of [<sup>3</sup>H]-thymidine (Y-axis) into Epo dependent HCD57 cells.

**Figure 19. Biological Activity of Muteins Adjacent to Helix D.** The top panel shows the region adjacent to the C-terminal boundary of Epo helix D with arrows indicating the positions where modifications were made to create muteins. The lower panel is a dose response curve representing the biological activity over a range of dosages (X-axis) of wild type Epo (■) and muteins adjacent to helix D K152A (◆), K154A (□), and Y156A (◇) as determined by the relative incorporation of [<sup>3</sup>H]-thymidine (Y-axis) into Epo dependent HCD57 cells.

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Figure 20. Comparison of Muteins With Increased Biological Activity. A dose response curve representing the biological activity of wild type Epo (\*), and muteins R143A (□) and K154A (♦) with increased biological activity relative to wild type Epo is shown over a range of dosages (X-axis) as determined by the relative incorporation of [<sup>3</sup>H]-thymidine (Y-axis) into Epo dependent HCD57 cells. n.

Figure 21. Comparison of Muteins With Increased Biological Activity in Non-malignant Cells. A dose response curve representing the biological activity of wild type Epo (\*), and muteins R143A (□) and K154A (♦) with increased biological activity relative to wild type Epo, is shown over a range of dosages (X-axis) as determined by the relative incorporation of [<sup>3</sup>H]-thymidine (Y-axis) into murine spleen cells.

Figure 22. Comparison of Muteins With Increased Biological Activity in a Human Cell Line. A dose response curve representing the biological activity of wild type Epo (x), and muteins R143A (□), and K154A (♦) with increased biological activity (relative to wild type Epo) is shown over a range of dosages (X-axis) as determined by the relative incorporation of [<sup>3</sup>H]-thymidine (Y-axis) into human Epo-dependent UT-7/Epo cell line (Komatsu, N. et al., *Cancer Res.* 51: 341-348 (1991)).

Figure 23. Comparison of Muteins with Increased Biological Activity in a Human Cell Line. A dose response curve representing the biological activity of wild-type Epo (□) and mutein N147A (♦) with increased biological activity (relative to wild-type Epo) is shown over a range of dosages (mU) (X-axis) as determined by the relative incorporation of [<sup>3</sup>H]-thymidine (Y-axis) into human Epo-dependent UT-7/Epo cell line.

Figure 24. Comparison of Muteins with Increased Biological Activity in a Human Cell Line. A dose response curve representing the biological activity of wild-type Epo (□) and mutein N147A (♦) with increased biological activity (relative to wild-type Epo) is shown over a range of dosages

(mU) (X-axis) as determined by the relative incorporation of [<sup>3</sup>H]-thymidine (Y-axis) into human Epo-dependent UT-7/Epo cell line.

**Figure 25. Comparison of Muteins with Increased Biological Activity in a Human Cell Line.** A dose response curve representing the biological activity of wild-type Epo (□) and mutein N147A (◆, □) with increased biological activity (relative to wild-type Epo) is shown over a range of dosages (mU) (X-axis) as determined by the relative incorporation of [<sup>3</sup>H]-thymidine (Y-axis) into human Epo-dependent UT-7/Epo cell line.

**Biological Activities of Helix A Muteins.**

**Figure 26. Comparison of Muteins with Increased Biological Activity in a Human Cell Line.** A dose response curve representing the biological activity of wild-type Epo (□) and mutein K20A with increased biological activity relative to the wild type Epo, is shown over a range of dosages (X-axis) as determined by the relative incorporation of [<sup>3</sup>H]-thymidine (Y-axis) into UT-7 Epo cells.

**Figure 27. Comparison of Muteins with Increased Biological Activity in a Human Cell Line.** A dose response curve representing the biological activity of wild-type Epo (□) and mutein Y49S with increased biological activity relative to the wild type Epo, is shown over a range of dosages (X-axis) as determined by the relative incorporation of [<sup>3</sup>H]-thymidine (Y-axis) into UT-7 Epo cells.

**Biological Activities of Helix B Muteins.**

**Figure 28. Comparison of Muteins with Increased Biological Activity in a Human Cell Line.** A dose response curve representing the biological activity of wild-type Epo (□) and mutein A73G with increased biological activity relative to the wild type Epo, is shown over a range of dosages (X-axis) as determined by the relative incorporation of [<sup>3</sup>H]-thymidine (Y-axis) into UT-7 Epo cells.

**Biological Activities of Helix D Muteins.**

**Figure 29. Comparison of Muteins with Increased Biological Activity in a Human Cell Line.** A dose response curve representing the

biological activity of wild-type Epo (□) and mutein K140A with increased biological activity relative to the wild type Epo, is shown over a range of dosages (X-axis) as determined by the relative incorporation of [<sup>3</sup>H]-thymidine (Y-axis) into UT-7 Epo cells.

5       *Figure 30a. Comparison of Muteins with Increased Biological Activity in a Human Cell Line.* A dose response curve representing the biological activity of wild-type Epo (□) and mutein R143A with increased biological activity relative to the wild type Epo, is shown over a range of dosages (X-axis) as determined by the relative incorporation of [<sup>3</sup>H]-thymidine (Y-axis) into UT-7 Epo cells.

10      *Figure 30b. Comparison of Muteins with Increased Biological Activity in a Mouse Cell Line.* A dose response curve representing the biological activity of wild-type Epo (□) and mutein R143A with increased biological activity relative to the wild type Epo, is shown over a range of dosages (X-axis) as determined by the relative incorporation of [<sup>3</sup>H]-thymidine (Y-axis) into HCD 57 cells.

15      *Figure 31. Comparison of Muteins with Increased Biological Activity in a Human Cell Line.* A dose response curve representing the biological activity of wild-type Epo (□) and mutein S146A with increased biological activity relative to the wild type Epo, is shown over a range of dosages (X-axis) as determined by the relative incorporation of [<sup>3</sup>H]-thymidine (Y-axis) into UT-7 Epo cells.

20      *Figure 32. Comparison of Muteins with Increased Biological Activity in a Human Cell Line.* A dose response curve representing the biological activity of wild-type Epo (□) and mutein N147A with increased biological activity relative to the wild type Epo, is shown over a range of dosages (X-axis) as determined by the relative incorporation of [<sup>3</sup>H]-thymidine (Y-axis) into UT-7 Epo cells.

25      *Figure 33a. Comparison of Muteins with Increased Biological Activity in a Human Cell Line.* A dose response curve representing the biological activity of wild-type Epo (□) and mutein K154A with increased

biological activity relative to the wild type Epo, is shown over a range of dosages (X-axis) as determined by the relative incorporation of [<sup>3</sup>H]-thymidine (Y-axis) into UT-7 Epo cells.

*Figure 33b. Comparison of Muteins with Increased Biological*

- 5 **Activity in a Mouse Cell Line.** A dose response curve representing the biological activity of wild-type Epo (□) and mutein K154A with increased biological activity relative to the wild type Epo, is shown over a range of dosages (X-axis) as determined by the relative incorporation of [<sup>3</sup>H]-thymidine (Y-axis) into HCD 57 cells.

- 10 *Figure 33c. Comparison of Muteins with Increased Biological Activity in Non-Malignant Mouse Cells.* A dose response curve representing the biological activity of wild-type Epo (□) and mutein K154A with increased biological activity relative to the wild type Epo, is shown over a range of dosages (X-axis) as determined by the relative incorporation of [<sup>3</sup>H]-thymidine (Y-axis) into primary murine spleen cells.

- 15 *Figure 34. Bioactivity of Epo Replacement Muteins.* The letters in the column on the left designate predicted helices (A, B, C, D) or interhelical loops (A-B, C-D). The 3'D designation (3' of D) in the table represents amino acids 153-166 which are at the carboxy-end of the D-helix. The amino acid of wild type human Epo, its position from the N-terminus, and its replacement according to the single amino acid letter code, are listed under the mutein column, e.g. S9A, represents a mutation from the wild-type human Epo at the amino acid serine of position number 9 to alanine. The three bioassay columns (human UT7 cell line, murine HCD57 cell line, and primary mouse spleen erythroid cells) show specific bioactivity of each mutein, expressed as a percentage of wild type human Epo bioactivity, with the background COS supernatant alone subtracted from the value. The mean and standard deviation of the bioassay values are listed for n ≥ 3 determinations. The numbers within the brackets indicate the number of separate COS cell transfections over (/) the number of separate bioassays that were performed.
- 20 NS: not secreted from COS 7 cells.
- 25
- 30

*Definitions*

In order to provide a clearer and more consistent understanding of the specification and claims, including the scope to be given such terms, the following definitions are provided.

- 5        *Administration.* The term "administration" is meant to include introduction of the Epo mutoins of the invention into an animal or human by any appropriate means known to the medical or veterinary art, including, but not limited to, injection, oral, enteral, and parenteral (e.g., intravenous) administration.
- 10      *Amino Acid Codes.* The most common amino acids and their codes are described in the following table:

- 20 -

Table 1

## Amino acid names and codes

Amino acid	Single letter code	Three letter code
Alanine	A	Ala
Arginine	R	Arg
Aspartic acid	D	Asp
Asparagine	N	Asn
Cysteine	C	Cys
Glutamic acid	E	Glu
Glutamine	Q	Gln
Glycine	G	Gly
Histidine	H	His
Isoleucine	I	Ile
Leucine	L	Leu
Lysine	K	Lys
Methionine	M	Met
Phenylalanine	F	Phe
Proline	P	Pro
Serine	S	Ser
Threonine	T	Thr
Tryptophane	W	Trp
Tyrosine	Y	Tyr
Valine	V	Val

*Animal.* The term "animal" is meant to include all animals whose production of red blood cells (erythrocytes) is dependent upon, or stimulated by, erythropoietin (Epo). Foremost among such animals are humans; however, the invention is not intended to be so limiting, it being within the contemplation of the present invention to treat any and all animals which may experience the beneficial effect of the Epo mutoins of the invention.

**Efficacious Amount.** An "efficacious amount" of an Epo mutein of the invention is an amount of such Epo mutein that is sufficient to bring about a desired result, particularly the stimulation of red blood cell production, especially upon administration to an animal or human.

5       **Erythrocyte.** The term "erythrocyte" is intended to refer to a red blood cell.

**Erythroid Precursor Cells.** By "erythroid precursor cells" is intended cells with a capability to proliferate and differentiate into erythrocytes that is dependent upon exposure or contact with Epo.

10      **Erythropoietin (Epo).** The term "erythropoietin", or "Epo" is primarily intended to refer to the mature form of this hormone. The terms "wild type Epo" and "native Epo" are used interchangeably to refer to Epo in its naturally occurring, unmodified form. Unless otherwise indicated, amino acid positions on the Epo protein referred to herein are made with reference  
15 to the mature, 166 amino acid human Epo sequence shown in Figure 6 and corresponding sequences from other species.

20      **Host Cell.** By "host" or "host cell" is intended the cell in which a gene encoding an Epo mutein of the invention is incorporated and expressed. An Epo mutein gene of the invention may be introduced into a host cell as part of a vector by transformation.

**Mutein.** By "mutein" is meant a mutant or modified protein with one or more modifications to its native amino acid sequence in the form of amino acid additions, deletions, or substitutions. An erythropoietin mutein refers to a modified erythropoietin protein.

25      **Pharmaceutically Acceptable Vehicle.** The term "pharmaceutically acceptable vehicle" is intended to include solvents, carriers, diluents, and the like, which are utilized as additives to preparations of the Epo muteins of the invention so as to provide a carrier or adjuvant for the administration of such Epo muteins.

30      **Transformation.** By "transformation" is intended the act of causing a host cell to contain a desired nucleic acid molecule, including either a native

Epo gene or a gene encoding an Epo mutein of the invention, not originally part of that cell using methods known in the art.

5        *Transfection.* By "transfection" is intended the introduction of a DNA or RNA vector carrying a desired nucleic acid molecule, including either a native Epo gene or a gene encoding an Epo mutein of the invention, into a host cell.

Vector. By "vector" is intended a DNA element used as a vehicle for cloning or expressing a desired gene, such as an Epo mutein gene of the invention, in a host.

10        *Detailed Description of the Preferred Embodiments*

This invention is drawn to modifications of Epo which enhance its biological activity. Due to their enhanced activity, the Epo muteins of the invention provide a preferable alternative to native Epo where it is used to stimulate red blood cell production (e.g. treatment of blood disorders, cell culture, etc.).

15        The present invention teaches a structural model of the Epo protein useful for identifying functional regions which may be modified to enhance biological activity. According to the invention, Epo is predicted to have a four anti-parallel amphipathic  $\alpha$ -helical bundle structure. Based upon this predicted structure, functionally important regions predicted to reside on the external surfaces of the helices (designated A-D, see Figures 9A-9B) are identified. In particular, the external surfaces of helices A and D, predicted to be involved in Epo receptor binding, are identified. According to the invention, these functionally important regions serve as targets for 20      modifications which may enhance biological activity. Increased activity 25      relative to the wild-type Epo can be found with amino acid substitutions in the A,B,C or D helix.

According to one aspect of the invention, Epo muteins are provided in which one or more of the amino acids within the predicted external surfaces

of helices A, B, C, D and 3' of D, or within the region immediately adjacent to the C-terminal end of helix D, have been replaced. In particular, amino acid substitutions at positions 20, 49, 73, 140, 143, 146, 147 and 154 of wild type Epo have been replaced. The inventors have discovered that substitution 5 of the amino acids normally occurring at these positions results in Epo muteins with significantly higher biological activity than wild type Epo proteins.

According to the invention, the amino acids at position 20 (typically alanine), 49 (typically serine), 73 (typically glycine), 140 (typically lysine), position 143 (typically arginine), position 146 (typically serine), position 147 10 (typically asparagine), and 154 (typically either lysine or threonine) of wild type Epo are preferably replaced with an alanine residue. In addition to alanine other amino acids with a chemical structure and properties similar to alanine, such as serine and threonine, are contemplated by the invention as suitable substitutes for achieving Epo muteins of the invention with enhanced 15 biological activity.

In another aspect of the invention, a portion of the predicted interloop region between helices A and B extending from amino acid 48-52 is identified as important to Epo function. According to the invention, this functionally 20 important region serves as another target for modifications which may enhance biological activity. The inventors have discovered that substitution of the amino acid at position 49 results in Epo muteins with significantly higher biological activity than wild type Epo proteins.

According to the invention, the amino acid at position 49 (typically tyrosine) of wild type Epo is preferably replaced with a serine residue. Other 25 amino acids with a chemical structure and properties similar to serine, such as alanine and threonine, are contemplated as suitable substitutes for achieving Epo muteins of the invention with enhanced biological activity.

The modifications taught by the present invention occur in regions that are highly conserved among Epo proteins from evolutionarily divergent species 30 (see Example I and Figure 6 in particular). These modifications are thus expected to be broadly applicable to Epo proteins which are substantially similar to the human Epo protein in the regions where the modifications occur,

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including, but not limited to, Epo proteins from monkey, mouse, rat, sheep, pig, cat, and dog.

Biological activity of Epo muteins can be determined by assaying the ability of these proteins to stimulate the proliferation of Epo responsive cells such as murine spleen cells (Krystal, G., *Exp. Hematol.* 11:649-60 (1983); Goldberg, M.A., *et al.*, *Proc. Natl. Acad. Sci. USA* 84:7972-7976 (1987)), murine Epo responsive murine erythroleukemia cells (Hankins, W.D., *et al.*, *Blood* 70:173a (1987)), and human Epo-dependent UT-7/Epo cells (Komatsu, N., *et al.*, *Cancer Res.* 51:341-348 (1991)).

When assaying for biological activity, it is important to recognize that, as taught by the present invention, high levels of biologically active Epo can cause terminal maturation and cessation of division of Epo responsive cells. Because of this possibly confounding phenomenon, it is important to determine biological activity based on a full dose response curve over a broad range of dosages which includes non-saturating levels of the particular Epo mutein being assayed.

Although the mechanism by which the modifications taught by the invention increase biological activity is not completely understood, one possibility is that these modifications enhance receptor binding affinity. This possibility is thought to apply particularly to the modifications at positions 20, 49, 73, 140, 143, 146, 147, and 154, which lie within a region predicted to be involved in Epo receptor binding according to the teachings of the present invention.

### ***Genes Encoding Epo muteins (Epo mutein genes)***

In addition to the Epo muteins themselves, genes encoding these muteins are also contemplated by the present invention. Genes encoding the Epo muteins of the invention may be made by modification of the native Epo gene using standard methods well known to those of skill in the art.

The native Epo gene may be obtained using a variety of standard techniques. For example, a DNA molecule corresponding to the known

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sequence of the Epo gene from a number of species may be artificially synthesized (See Example I and Figure 4). The Epo gene may also be isolated from a cDNA or genomic library using nucleic acid probes based on available Epo DNA sequence information and standard hybridization techniques as taught in U.S. Pat. No. 4,703,008, issued Oct. 27, 1987, which is herein incorporated by reference (See also Jacobs *et al.*, *Nature* 313: 806-810 (1985)). Alternatively, amplification of Epo gene sequences by polymerase chain reaction (PCR) may be accomplished using primers based on available sequence data as taught in Example I.

Once obtained, the wild type Epo coding sequence can be altered to encode an Epo mutein of the invention using standard oligonucleotide-directed *in vitro* mutagenesis techniques (See Zoller, M. and Smith, M., *Methods in Enzymology* 100: 468-500 (1983); Dente, L. *et al.*, *Nucl. Acids Res.* 11: 1645 (1983); Kunkel, T.A., *et al.*, *Meth. Enzymol.* 154:367-382 (1987); Vandeyar, M. *et al.*, *Gene* 65: 129 (1988)). These techniques generally involve hybridization of an oligonucleotide carrying the desired alteration to a single stranded target DNA. This is followed by complementary strand synthesis of the target DNA which incorporates the oligonucleotide carrying the desired alteration. The complementary strand containing the desired alteration is then propagated in a bacterial host cell. Specific application of this type of technique to modify the Epo gene is described in Examples II and III and in Australian Patent Publication No. AU-A-59145/90 by Fibi *et al.* and in U.S. Pat. No. 4,835,260 by Shoemaker, both of which are herein incorporated by reference.

25

### *Expression of Epo mutein Genes*

Genes encoding Epo muteins of the invention can be introduced and expressed in a selected host cell system using conventional materials and techniques. DNA elements such as promoters, enhancers, polyadenylation sites, transcription termination signals, and the like should be associated with

the Epo mutein coding sequence so as to promote and control expression of the Epo mutein. The specific regulatory elements used will depend upon the host cell system selected for expression, whether secretion of the protein is desired, and other considerations readily apparent to one of skill in the art.

5        Various vectors may be employed as vehicles for the introduction and expression of Epo mutein genes in a host cell. Such vectors useful in the different host cell types are well known and include, for example, the mammalian expression vectors pSG5 (Stratagene), p-RK1 (Genetics Institute), p-SVK3 (Pharmacia), p-EUK-C1 (Clontech), pCDM (Invitrogen), pc DNAI 10 (Invitrogen), and the bacterial expression vectors pFLAG-1 (IBI), all pET system plasmids (Novagen), pTrcHis (Invitrogen), the pGEX series (Pharmacia), and pKK 233-2 (Clontech). These vectors may be maintained as episomes in the host cell or they may facilitate integration of the Epo mutein gene into the host cell genome, or both. Vectors may also include 15 other useful features, such as genes which allow for the selection or detection of cells in which they have been successfully introduced.

Host cells suitable for expression of the Epo muteins of the invention include, but are not necessarily limited to, *E. coli*, yeast, insect cells, plant cells, and a variety of mammalian cell types, including in particular Chinese 20 Hamster Ovary (CHO) cells, Cos7 cells, Cos1 cells, baby hamster kidney cells, and CV1 cells. Epo mutein genes can be introduced into a host cell using standard transformation or transfection techniques.

Host cells which provide for glycosylation are preferred for production 25 of Epo muteins to be used *in vivo* since the carbohydrate structure of Epo is important for optimal biological activity *in vivo* (See Dordal, M.S. *et al.*, *Endocrinology* 116(6): 2293-2299 (1985)).

The Epo muteins of the invention may be recovered and purified from host cells in which they are expressed using known methods such as immunoaffinity chromatography with antibodies to human Epo.

*Therapeutic Use of Epo muteins  
of the Invention*

The Epo muteins of the invention are suitable for therapeutic use in animals, particularly humans, and may be used in the same manner as wild type Epo, except that lower dosages will be required to achieve the same level of biological activity.

Administration of the Epo muteins of the invention may be accomplished by any of the methods known to the skilled artisan, provided that the method effectively places the Epo mutein in the appropriate environment to exhibit its activity (i.e. in contact with erythroid precursor cells). A preferred method is by parenteral routes, including intravenous and subcutaneous administration.

Administration will ordinarily include an efficacious amount of the Epo mutein supplied in a pharmaceutically acceptable vehicle. The amount of Epo mutein which is efficacious will be determined by a trained professional depending upon a number of considerations, including the condition being treated and its severity, the sex and body weight of the subject being treated, the method of administration, and the presence of compositions in the pharmaceutically acceptable vehicle which affect Epo activity.

In any event, the efficacious amount of a Epo mutein as taught by the invention will be significantly less than the corresponding amount of unmodified Epo needed to achieve the same degree of efficacy. The difference between the efficacious amount of the Epo mutein and the analogous amount of unmodified Epo will correspond with the increased biological activity exhibited by the Epo mutein. It is contemplated that an efficacious amount of an Epo mutein of the invention will be as low as 10-fold less than native Epo, or as low as about 5-10 units/kg body weight for treating chronic renal failure patients or about 10-30 units/kg body weight for treating HIV-infected patients with serum Epo levels less than or equal to 500 mU/ml

(milliunits/milliliter) who are receiving a dose of AZT equal to, or less than, 4200 mg/week.

Since the Epo muteins of the invention can be used effectively at lower dosages relative to wild type Epo, their use may reduce potential adverse effects associated with administration of Epo such as exacerbation of hypertension, seizures, headaches, tachycardia, nausea, clotted vascular access, shortness of breath, hyperkalemia, and diarrhea (see PDR, 1993 edition, at pp. 603-604).

Further description of methods and materials useful in the recombinant production of the Epo muteins of the invention and their use is provided in the teachings of U.S. Patent No. 4,835,260 by Shoemaker and U.S. Patent No. 4,703,008 by Lin, both of which are herein incorporated by reference in their entirety.

The invention may be better understood and appreciated by reference to the following examples. These examples are provided merely for illustrative purposes and should not be considered in any way as limiting the scope of the claimed invention.

#### **EXAMPLE I**

##### **20           *Erythropoietin Structure-Function Relationships: High Degree of Sequence Homology Among Mammals***

##### ***Summary***

In order to investigate structure-function relationships of erythropoietin (Epo), we have obtained cDNA sequences that encode the mature Epo protein of a variety of mammals. A first set of primers, corresponding to conserved 25 nucleotide sequences between mouse and human DNAs, allowed us to amplify by PCR intron 1-exon 2 fragments from genomic DNA of hamster, cat, lion, dog, horse, sheep, dolphin and pig. Sequencing of these fragments permitted the design of a second generation of species-specific primers. RNA was

prepared from anemic kidneys and reverse-transcribed. Using our battery of species-specific 5' primers, we were able to successfully PCR amplify Epo cDNA from Rhesus monkey, rat, sheep, cat and pig. Deduced amino acid sequences of mature Epo proteins from these animals, in combination with known sequences for human, Cynomolgus monkey and mouse, showed a high degree of homology, which explains the biological and immunological cross reactivity that has been observed in a number of species. Human Epo is 91% identical to monkey Epo, 85% to cat Epo and 80-82% to pig, sheep, mouse and rat Epos. There was full conservation of a) the disulfide bridge linking the NH<sub>2</sub> and COOH termini; b) N-glycosylation sites; and c) predicted amphipathic  $\alpha$ -helices. In contrast, the short disulfide bridge (C29/C33 in human) is not invariant. Cys33 was replaced by a Pro in rodents. Most of the amino acid replacements were conservative. The C-terminal part of the loop between the C and D helices showed the most variation, with several amino acid substitutions, deletions and/or insertions. Calculations of maximum parsimony for intron 1/exon 2 sequences as well as coding sequences enabled the construction of cladograms that are in good agreement with known phylogenetic relationships.

#### INTRODUCTION

A large number of early physiologic studies have established extensive, though not complete, biological cross-reactivity between Epos of man and a number of other mammals including mouse, rat, sheep and rabbit (Jelkmann, W., *Physiol. Rev.* 72:449 (1992); Garcia, J.F. et al., *Proc. Soc. Exp. Biol. Med.* 112:712-714 (1962); Zanjani, E.D. et al., *Proc. Soc. Exp. Biol. Med.* 131:1095-1098 (1969); Biljanovic-Paunovic, L. et al., *Period. Biol.* 90:421-427 (1988)). In contrast, non-mammalian vertebrates (amphibians (Rosse, W.F., et al., *Blood* 22:66-72 (1963); Pinder, A. et al., *J. Exp. Biol.* 105:205-213 (1983)), birds (Rosse, W. et al., *Blood* 27:654-661 (1966); Black, C.P. et al., *Respirat. Physiology* 39:217-239 (1980)), fish (Zanjani,

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E.D., *et al.*, *Science* 33:573 (1969)) have erythropoietic hormones that fail to cross-react with mammalian erythroid cells, and vice-versa (Rosse, W.F., *et al.*, *Blood* 22:66-72 (1963); Pinder, A. *et al.*, *J. Exp. Biol.* 105:205-213 (1983); Rosse, W. *et al.*, *Blood* 27:654-661 (1966)).

5        Thus far, the Epo genes of man (Jacobs, K. *et al.*, *Nature* 313:806-10 (1985); Lin, F.-K. *et al.*, *Proc. Natl. Acad. Sci. USA* 82:7580-7584 (1985)),  
a monkey (*Macaca fascicularis*) (Lin, F.-K. *et al.*, *Gene* 44:201-209 (1986))  
and a rodent, the mouse (McDonald, J.D. *et al.*, *Mol. & Cell Biol.* 6:842-848  
(1986); Shoemaker, C.B. *et al.*, *Mol. & Cell Biol.* 6:849-858 (1986)) have  
10      been cloned, sequenced, and expressed. In view of the marked cross-  
reactivity between mammalian Epos, it is not surprising that there is a high  
degree of sequence homology in the coding region of the mature secreted  
proteins. In keeping with their close phylogenetic relationship, human and  
monkey Epos are 94% and 91% identical in nucleotide and amino acid  
15      sequence respectively. In contrast, human and mouse Epos are 76% identical  
in nucleotide sequence and 80% identical in amino acid sequence.

20       Direct information on the three-dimensional structure of Epo is not yet  
available. Insights into structure-function relationships of Epo can be gained  
from the analyses of a more complete set of animal sequences. Such  
information could be useful for sequence-based computer modeling of three-  
dimensional structure. Moreover, a larger data base would permit the  
identification of highly conserved domains that are likely to be crucial to  
folding and/or biological function. Finally, comparative amino acid and  
nucleotide sequence information on Epo provides additional data for  
25      investigating phylogenetic relationships.

In this Example, the use of PCR based techniques for obtaining the full  
coding sequences of Epos of Rhesus monkey, rat, sheep, pig and cat, as well  
as partial sequences from four other species is demonstrated.

*Materials and Methods*

*Animal Samples.* Human hepatoma Hep3B cell line and multiple kidney-derived cell lines from hamster (BHK), sheep (MDOK), pig (LLC-PK1), dog (MDCK), cat (CRF-K), lion (PAL1-K) and spotted dolphin (SP1-K) 5 were obtained through the American Type Culture Collection (American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852). Monolayer cells were grown in 100 x 20 mm tissue culture dishes using recommended media and maintained in a humidified 5% CO<sub>2</sub>/95% air incubator at 37°C. In some experiments, cells were made hypoxic by 10 overnight incubation in 1% O<sub>2</sub>, 5% CO<sub>2</sub>, 94% N<sub>2</sub> at 37°C.

Poly A + RNA prepared from Rhesus monkey kidney was purchased from Clontech (Palo Alto, CA). Kidneys from anemic cat and horse were obtained from veterinarians at Tufts School of Veterinary Medicine and the University of Nebraska, respectively. Anemia was induced by three 15 consecutive daily intraperitoneal injections of phenylhydrazine (60 mg/kg body weight) into male Sprague-Dawley rats (SD-strain) and by repeated bleeding of the sheep and the pig. Kidneys were aseptically removed after induction and stored immediately in liquid nitrogen.

A 4 kb genomic human Epo clone (g Epo4) was provided by Genetics 20 Institute (Boston, MA).

*DNA and RNA Preparations.* DNAs from cell lines or homogenized kidneys were extracted using pancreatic RNAase/SDS/proteinase K following a procedure modified from Blin and Stafford (Blin, N. *et al.*, *Nucleic Acids Res.* 3:2303-2308 (1976)).

Kidneys were homogenized in 4 M guanidine isothiocyanate (10 ml/g 25 of frozen organ), containing 0.1 M beta-mercaptoethanol and 10% N-laurylsarcosine. Total RNAs were isolated by centrifugation over 5.7 M CsCl (Chirgwin, J.M. *et al.*, *Biochemistry* 18:5294 (1979)). After ethanol precipitation, the samples were resuspended in diethyl pyrocarbonate-treated 30 water and stored at -70°C. Confluent monolayer cells were washed twice with

sterile phosphate buffered saline and directly lysed in the 4 M guanidine isothiocyanate solution. Total RNAs were isolated as described above for the kidneys.

RNA samples were first converted into single strand cDNA. 2 to 4  $\mu$ g of total RNA from kidney or 500 ng to 1  $\mu$ g of total RNA from cultured cells were denatured at 68°C in the presence of 2  $\mu$ g of oligo dT<sub>(15)</sub>. Reverse transcription was carried out in a 20  $\mu$ l final volume, containing 50 mM Tris-HCl pH 8.3, 6 mM MgCl<sub>2</sub>, 40 mM KCl, 10 mM DTT, 500  $\mu$ M dNTPs, 20  $\mu$  u RNAsin (Promega, Madison, WI) and 20 u of avian myeloblastosis virus reverse transcriptase (AMV-SuperRT, Molecular Genetic Resources, Tampa, FL). The reaction was allowed to proceed at 37°C for 20 minutes, then at 42°C for one hour. Inactivation of the enzyme was performed at 100°C for 10 minutes. cDNA samples were stored at -70°C.

*Polymerase Chain Reaction.* 20 to 100 ng of genomic-DNA or 0.5 to 1  $\mu$ l of the RT-reaction were used as templates for amplification.

Standard PCR reactions were performed in a 100  $\mu$ l volume containing 50 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.01% w/v gelatin, 200  $\mu$ M dNTPs, 30 pM of each sense (5') and antisense (3') primers and 0.5 units of Taq DNA polymerase (Perkin Elmer Cetus, Emeryville, CA). After an initial denaturation step for 5 minutes at 95°C, routinely 35 cycles of PCR were performed on the DNA Thermal Cycler (Perkin Elmer Cetus). The denaturation step of each cycle was carried out at 94°C for 1 min. For each sample and/or the primer pair used, annealing temperatures were optimized (from 45°C to 60°C) for a 2-minute reaction. A 3-minute elongation step at 72°C ended each cycle.

Amplification products were analyzed on 1 to 3% agarose gel-TAE. Nu Sieve or Seaplaque agaroses (FMC., Rockland, ME) were used for preparative purposes. Specific PCR products were recovered from the gel, by the use of the Gene Clean II kit (Bio 101, La Jolla, CA), by Spin X centrifugation (Costar, Cambridge, MA) or by standard phenol/chloroform extractions of melted gel slices.

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*Subcloning and Sequencing.* Small (250-330 bp) amplified genomic fragments were directly subjected to automated sequencing, using the PCR primers as DNA sequencing primers.

PCR-generated cDNA products were blunt-ended using Klenow fragment and 5' phosphorylated by T4 DNA kinase (Boehringer Mannheim, Indianapolis, IN). They were then cloned into the SmaI site of the phagemid pBluescript II SK (Stratagene, La Jolla, CA). The double-stranded plasmid was sequenced using the SK and KS sequencing primers.

In all cases, Sequencing reactions were carried out on an Applied Biosystems 373A Automated DNA Sequencer utilizing ABI's DyeDeoxy Terminator Sequencer kit (Foster City, CA) and thermal cycling with Taq DNA polymerase (Promega, Madison, WI) as previously described (Tracy, T.E. *et al.*, *Biotechniques* 11:68-74 (1991)). To avoid errors, for each species, samples derived from different amplification/cloning experiments were prepared and both strands were sequenced.

*Mammalian Expression; Bioactivity.* Vectors containing full-length mammalian Epo coding sequences were subcloned into pSG5 plasmid (Pharmacia, Piscataway, NJ) and were transiently expressed in Cos7 cells. 72-hour supernatants were tested for their ability to sustain cellular proliferation of the Epo-dependent HCD57 cell line (Sawyer, S.T. *et al.*, *Blood* 72 (suppl. 1): 132a (1988)).

### *Results and Discussion*

Because of the known biological cross-reactivity between various mammalian Epos and the presumption of strong homology in the coding sequences, a logical cloning strategy would be to screen cDNA libraries with moderate to low stringency. However, Epo mRNA is expressed at barely detectable levels in all tissues except in hypoxic kidney and liver (Goldberg, M.A. *et al.*, *Proc. Natl. Acad. Sci. USA* 84: 7972-7976 (1987); Fandrey, J. *et al.*, *Blood* 81: 617-623 (1993)). Therefore it would be necessary to

generate libraries for each of the species of interest. Our primary research interest is in structure-function relationships of Epo and therefore we require full coding sequences of the mature Epo protein, rather than full cDNA sequences. Accordingly, we elected to employ a PCR cloning strategy  
5 predicated on known strong homologies in Epo cDNA sequences that flank the region encoding the mature protein. There was sufficient conservation of sequence around the NCO1 site in the 3' untranslated region (UTR) that a single primer could be used to amplify full length mature Epo coding sequence in rat, sheep, pig and cat as well as dog and rabbit (data not shown).  
10 However, we were unsuccessful in finding a universal 5' primer and therefore had to generate species specific sequences from amplifications of genomic DNA. This two stage PCR strategy proved to be satisfactory in generating highly accurate sequence information with a minimum expenditure of time and resources. In all instances, there was full agreement between sequences of  
15 complementary strands.

*Preparation of Mammalian Epo cDNAs*

*Primer Design for PCR Amplification of Epo.* The sequences of Epo genes from three species have been already published: two primates, human (Jacobs, K. et al., *Nature* 313:806-810 (1985); Lin, F.-K. et al., *Proc. Natl. Acad. Sci. USA* 82:7580-7584 (1985)) and Cynomolgus monkey (*Macaca fascicularis*) (Lin, F.-K. et al., *Gene* 44:201-209 (1986)) and a rodent, the mouse (*Mus muscuius*) (McDonald, J.D. et al., *Mol. & Cell Biol.* 6:842-848 (1986); Shoemaker, C.B. et al., *Mol. & Cell Biol.* 6:849-858 (1986)). There is substantial homology at the nucleotide level not only in the coding sequences, but also in portions of introns and in 5' and 3' untranslated sequences of exons 1 and 5, respectively. A number of primers, corresponding to conserved nucleotide sequences between mouse and-human, were synthesized and tested for their ability to produce genomic-PCR fragments from a wide variety of mammalian cell lines. A 4 kb human

genomic clone and genomic DNA extracted from the human hepatoma cell line Hep3B were used as control templates for the PCR amplifications.

In particular, two pairs of primers, IV1/EX2R and EX5/NCO1 (Figure 1), directed the amplifications of fragments of 330 and 250 bp respectively from genomic DNA of all human and mammalian cell lines that we investigated. IV1 is a 25-mer oligonucleotide localized in intron 1, 216/217 bases upstream from the start of exon 2. Human and mouse are identical except that the human sequence contains an extra G nucleotide at position 996. IV1 primer corresponding to the mouse sequence was synthesized. The 23-mer EX2R ends 28 nucleotides upstream from the 3' end of exon 2. EX5 is a 22-mer, beginning 34 bases downstream from the 5' end of exon 5. NCO1 represents a 20 bp, 100% conserved DNA fragment, starting 112/113 bases downstream from the TGA stop codon and contains the unique NCO1 site present in the human and mouse gene.

An ATG primer was also synthesized, corresponding to a 20 oligonucleotide stretch, extending both 5' and 3' from the initiator methionine codon. This sequence is also totally conserved between primates and mouse. While combinations of exonic primers (ATG1EX2R, EX5/NCO1 and ATG/NCO1) were able to direct amplification from cDNA prepared from RNA of the human Epo-producing cell line Hep3B, no amplification was obtained on cDNA prepared from uninduced or hypoxia-induced BHK, MDOK, LLC-PK1, MDCK, CRF-K, PAL1-K and SP1-K cells (results not shown). Therefore these renal cell lines apparently do not express Epo mRNA, either constitutively or after hypoxic stress, at a level detectable even by RT-PCR. Furthermore, no Epo protein was detectable by radioimmunoassay in the supernatants of cells maintained overnight in 1% O<sub>2</sub>, 5% CO<sub>2</sub>, 94% N<sub>2</sub> at 37°C (Goldberg, M.A. et al., *Proc. Natl. Acad. Sci. USA* 84:7972-7976 (1987)).

The amplification of species specific IV1/EX2R and EX5/NCO1 genomic fragments allowed us to design a strategy shown in Figure 2 for obtaining cDNA clones containing the complete coding sequence of mature

Epo protein from various mammals. The IV1/EX2R primer pair resulted in a genomic fragment (~ 330 bp) corresponding to the 3' third of the first intron and 80% of exon 2, encoding the COOH-end of the propeptide and the NH<sub>2</sub>-terminal amino acids of the mature protein. EX5/NCO1 primers amplified a segment of exon 5, containing the COOH-end of the Epo protein and a 3' untranslated sequence of about 140 bp downstream of the stop codon: DNA sequencing of these 5' and 3' fragments permitted, if necessary, the design of a second generation of species-specific primers (SP), localized outside the coding part of the mature protein (i.e., in the sequences encoding the propeptide and the 3' untranslated region).

*Amplification of Partial Intron 1/ Exon 2 Genomic Clones.* We first explored the efficacy of the IV1 and EX2R primers for amplification of genomic fragments from different purified DNA templates. In all the reactions, fragments of predicted size were the main (and, when using the most stringent annealing temperature, often the only) products detected by agarose gel electrophoresis. The (direct) sequences of these PCR-generated fragments are presented in Figure 3. The nucleotide alignment includes 6 different orders of mammals: one primate, human (*Homo sapiens*); two artiodactyls, sheep (*Ovis aries*) and pig (*Sus scrofa*); one perissodactyl, horse (*Equus caballus*); one cetacean, spotted dolphin (*Stenella plagiodon*); three carnivores, dog (*Canis familiaris*), cat (*Felis catus*) and lion (*Panthera leo*); two rodents, mouse (*Mus musculus*) and hamster (*Cricetus cricetus*). Cat and lion exhibited an almost complete sequence identity, with only one T to G nucleotide substitution near the 5' end of the amplified portion of the first intron.

The ability of the intronic IV1 to anneal to genomic DNA from various species and, in combination with EX2R, to amplify PCR fragments of identical size, demonstrated a high degree of conservation of sequence and position between mammals. This suggests that IV1 sequence may be involved to some extent in the regulation of the Epo gene expression. The comparison of IV1/EX2R sequences also revealed two remarkably conserved intronic

sequences: AT(T/A)GAATGAA(G/C)GC [SEQ. ID NO: 1] (nucleotides 1046 to 1058 in the human gene) and A(A/T)GGTN(G/C)GGG [SEQ. ID NO: 2] (nucleotides 1085 to 1094).

*Amplification of Partial Exon 5 Fragments from Genomic DNAs.*

5 PCR reactions were also performed applying the EX5/NCO1 primer pair to various purified genomic DNAs. As previously observed for the amplification of IV1/EX2R, in all the tested samples a unique main band of about 250 bp (as predicted for human and mouse genes) was detected on analytical agarose gel electrophoresis. Direct DNA sequencing of these fragments demonstrated  
10 that the bulk of the purified PCR product corresponded to the expected Epo exon 5 sequence. However, for some DNA samples, purified from horse kidney and several cell lines (BHK and LLC-PK1 in particular), unknown sequences were present to various extents. These minor contaminating PCR products generated extraneous peaks in the sequence data, resulting in  
15 ambiguities at several positions. Nevertheless, computer-edited analyses of generated 3' non-coding sequences were sufficient to design, if necessary, specific primers with greater than 95 % accuracy (data not shown).

*Cloning of Partial cDNAs Encoding the Complete Mature Epo Protein.* As we previously mentioned, we were unable to obtain any  
20 amplification with cDNAs prepared from renal-derived mammalian cell lines. Therefore, we tested our battery of primers on reverse-transcribed RNAs prepared from kidney of several mammals. We have successfully amplified Epo cDNA from: one primate, Rhesus monkey (*Macaca mulatta*), one carnivore, cat (*Felis catus*), one rodent, rat (*Rattus norvegicus*) and two  
25 artiodactyls, pig (*Sus scrofa*) and sheep (*Ovis aries*). The aligned nucleotide sequences are presented in Figure 3. They have been submitted to GenBank and have been assigned accession numbers.

Rhesus monkey, rat and sheep cDNAs were amplified using the ATG/NCO1 primer-pair combination. Pig and cat sequences were obtained  
30 by the use of a 5' 24-mer specific primer (SP1-5' TGCTTCTGCTATCTTGCTGCTGC 3') [SEQ. ID NO: 3]. IV1/EX2R

sequencing demonstrated that this exon 2 sequence was shared by the two species. In all PCR amplifications, NC01 was used as the reverse primer. Conservation of the NCO1 sequence among mammals suggests a potential biological role of this nucleotide sequence (perhaps in modulating the stability 5 of Epo mRNA) (Rondon, I. J. *et al.*, *J. Biol. Chem.* 266:16594-16598 (1991)). Considerable homology is also found in the sequenced 3' untranslated segment of exon 5 (Figure 4).

When compared with human Epo, the overall percentages of nucleotide identity are, respectively: 93.5 for the Rhesus monkey, 86.7 for the cat, 85.7 10 for the pig, 83 for the sheep and 75.5 for the rat. Rhesus monkey and Cynomolgus monkey (Lin, F.-K. *et al.*, *Gene* 44:201-209 (1986)) were 99.6% identical. Mouse (McDonald, J.D. *et al.*, *Mol. & Cell Biol.* 6:842-848 15 (1986); Shoemaker, C.B. *et al.*, *Mol. & Cell Biol.* 6:849-858 (1986)) and rat nucleotide sequences showed greater than 93% identity. The two sequenced artiodactyls, pig and sheep, showed 88% identity.<sup>1</sup>

#### *Comparison of Mammalian Epo Primary Sequences*

*Propeptides.* The predicted amino acid propeptide sequences of several mammals are presented in Figure 5. In the primates, there was strong conservation of the sequences of deduced propeptides, with only two amino 20 acid (aa) substitutions at positions -11 (Leu in humans vs. Val in both monkeys) and -2 (Leu in humans vs. Pro in both monkeys). Expression of Cynomolgus monkey Epo gene in cultured mammalian cells resulted in the production of mature monkey Epo that was elongated at the N-terminus by three additional residues: Val-Pro-Gly (Lin, F.-K. *et al.*, *Gene* 44:201-209 25 (1986)). As the Rhesus monkey has the same substitutions, an identical site

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<sup>1</sup> Several PCR attempts on total RNA purified from the horse's kidney were unsuccessful, even when using 5' and 3' specific equine primers (SP), presumably because analysis on 1% formaldehyde agarose gel demonstrated the degradation of the RNA.

of cleavage is likely. The Leu to Pro amino acid replacement at -2 is probably responsible for the differential activity of the signal peptidase observed between man and monkeys. The three rodents, mouse, rat, and hamster, were found to have identical propeptide amino acid sequences.

5       *Mature protein.* A comparison of the three previously published and our five deduced amino acid sequences of mature Epo proteins is shown in Figure 6. Erythropoietin is highly conserved among mammals. The amino acid alignment showed that more than 63% of the molecule is composed of invariant amino acids (106 residues) (Figure 6A). Most of the observed  
10 substitutions are conservative, involving residues with similar physical and chemical properties. The calculated percentages of sequence identity are shown in Table II below.

15

**TABLE II**  
**Degree of conservation among mammals**  
**of the mature Epo proteins**

20

		% identity
Primates	Man/Rhesus Monkey	90.5
	Rhesus/Cynomolgus	98.8
Rodents	man/mouse	79.8
	man/rat	82.1
	mouse/rat	93.5
Artiodactyls	man/sheep	81.0
	man/pig	81.6
	sheep/pig	88.7
Carnivores	man/cat	84.5

There is 100% conservation of Cys7 at the N-terminal portion of the polypeptide and Cys161 near the C-terminus. This disulfide bridge is essential

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to the formation of a stable and functional cytokine (Wang, F.F. *et al.*, *Endocrinology* 116:2286-2292 (1985)). In contrast, while Cys29 is invariant in all the species we examined, Cys33 is present only in primates, sheep, pig and cat, but not in the two rodents where there is a proline. The lack of 5 functional importance of this short disulfide loop is underscored by muteins in which tyrosine substitutions at each or both of these sites have no effect on Epo's biological activity (See Example II).

There is also 100% conservation of all three asparagine residues responsible for N-linked glycosylation. Even though Epo, deprived of its 10 carbohydrate either by enzymatic cleavage (Takeuchi, M. *et al.*, *J. Biol. Chem.* 265:12127-12130 (1990)) or by production in bacteria (Nahri, L.O. *et al.*, *J. Biol. Chem.* 266:23022-23026 (1991)) has full *in vitro* biological activity, survival of the hormone in the circulation depends upon N-linked glycosylation (Spivack, J.L. *et al.*, *Blood* 73:90-99 (1986)). In contrast, the 15 O-linked glycosylation site (Ser126 in human Epo) is not essential for biological function since it is missing in mouse and rat Epos.

As shown in Figure 6, there is very high conservation of sequence in regions which, by algorithms based on primary structure, are predicted to be alpha helices (See Example II). The few differences in sequence within these 20 helical regions are conservative replacements. Indeed, some sites within these predicted alpha helices are conserved in corresponding helices of other cytokines (Manavalan, P. *et al.*, *J. Prot. Chem.* 11:321-331 (1992)). In contrast, regions predicted to be inter-helical loops are less well conserved, particularly the 14 residue stretch between residues 116 and 130 of human 25 Epo. In Example II a mutein with a deletion of residues 111 through 119 is shown to have normal stability and biological activity, whereas a deletion of residues 122 through 126 fails to produce a detectable protein, probably owing to markedly impaired stability.

Similar overall 4  $\alpha$ -helical bundle structure exists or is predicted for 30 several other cytokines/hormones, such as growth hormone (GH), granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-3, IL-4 and IL-5 (Bazan,

J.F. *et al.*, *Immunol. Today* 11: 350-354 (1990)). Like Epo, GH exhibits a high degree of primary sequence conservation and biological crossreactivity between mammals. The mouse, rat, pig and sheep proteins show about 80% amino acid identity with human GH. In other cytokines, however, there is  
5 more sequence diversity among species. For example, the human and murine forms of IL-5, GM-CSF and IL-3 show respectively only 69, 67 and 26% amino acid homology. Furthermore, unlike Epo or GH, those proteins lack inter-species biological cross-reactivity.

*Expression of the Mammalian Epo Hormones.* Mammalian Epo  
10 cDNAs were subcloned into the pSG5 plasmid and transiently expressed in the monkey Cos7 cell line. Supernatants of the transfected cells were able to sustain the cellular proliferation of the murine Epo-dependent HCD57 cell line, demonstrating the biological cross-reactivity between species. As the human Epo antisera used in our radioimmunoassay bind to Epos from other species  
15 with variable affinity, the amount of produced protein was difficult to determine accurately. Experiments are in progress to further characterize the relative affinities of the various mammalian erythropoietins toward murine and human Epo receptors.

*Phylogenetic Analyses of Epo Sequences.* Nucleotide sequences  
20 encoding the full length mature Epo protein were analyzed by the maximum parsimony method (Czelusniak, J. *et al.*, *Meth. Enzymol.* 183:601-615 (1990); Stanhope, M.J. *et al.*, *Mol. Phylog. Evol.* 1:148-160 (1992)). An "all trees" set of computer programs determined the parsimony length of each of the 945 unrooted trees formed by the seven cDNA sequences (the terminal taxa)  
25 (Figure 4) and, on ordering those 945 trees according to increasing length, identified the minimum number of extra nucleotide substitutions needed to break up each group in the maximum parsimony (lowest length) tree. As shown in Figure 7 and 7A, the mouse and rat are strongly grouped, as are the human and monkey. The pig and sheep are more weakly grouped, in accord  
30 with accepted phylogenetic views, which place the divergence of these animals about 55-60 million years ago. As expected with the parsimony reconstruction

on only the codon sequences, silent base substitutions occurred much more readily than amino acid replacements (Figure 7B).

The Epo intron 1-exon 2 sequences shown in Figure 3 were also analyzed by the maximum parsimony method. Figure 8 shows strength of grouping results based on all 135,135 unrooted trees formed by nine terminal taxa (in this case, cat and lion sequences, which are nearly identical, were treated as a single taxon), and Figure 8A shows a phylogenetic tree that combines maximum parsimony with established phylogenetic evidence. As the most parsimonious tree (Figure 7) for these relatively short sequences failed to join dog to the feloid (cat and lion) branch (reflecting the fact that the ancestral split between canoids and feloids traces back to the early carnivores at about 60 million years ago), the phylogenetic tree shown in Figure 7A is a near most parsimonious tree in which dog joins the feloids. The rodents (mouse and hamster) are strongly grouped, as are the feloids (cat and lion), and at more moderate strength the cetacean (dolphin) is grouped with the artiodactyls (sheep and pig). There are other molecular data as well as some paleontological data that depict cetaceans originating from early artiodactyls (Czelusniak, J. *et al.* *Meth. Enzymol.* 183:601-615 (1990); Czelusniak, J. *et al.*, In "Current Mammalogy", Volume 2, 3rd ed., H. H. Ganoways, pp. 545-572, Plenum Publ. Corp. (1990); Irwin, D.M. *et al.*, *J. Mol. Evol.* 32:128-144 (1991); Gingreich, P.D. *et al.*, *Science* 249:154-157 (1990); Novacek, M.J., *Nature* 356:121-125 (1992)).

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### **EXAMPLE II**

#### ***Structure Function Relationships of Erythropoietin: Muteins that Test a Model of Tertiary Structure***

##### ***Summary***

5        On the basis of its primary sequence and the location of its disulfide bonds, we propose a structural model of the erythropoietic hormone erythropoietin (Epo) which predicts a 4  $\alpha$ -helical bundle motif, in common with other cytokines. In order to test this model, site directed mutants were prepared by high level transient expression in Cos7 cells and analyzed by a  
10      radio-immune assay and by bioassays utilizing mouse and human Epo dependent cell lines. Deletions of 5 to 8 residues within predicted  $\alpha$  helices resulted in the failure of export of the mutant protein from the cell. In contrast, deletions at the N-terminus ( $\Delta$ 1-6), the C-terminus ( $\Delta$ 163-166), or in predicted interhelical loops (AB:  $\Delta$ 32-36,  $\Delta$ 53-57; BC:  $\Delta$ 78-82; CD:  
15       $\Delta$ 111-119) resulted in the export of immunologically detectable Epo muteins that were biologically active. The mutein  $\Delta$ 48-52 could be readily detected by RIA but had markedly decreased biological activity. However, replacement of each of these deleted residues by serine resulted in Epo muteins with full biological activity. Replacement of Cys29 and Cys33 by  
20      tyrosine residues also resulted in the export of fully active Epo. Therefore this small disulfide loop is not critical to Epo's stability or function. The properties of the muteins that we tested are consistent with our proposed model of tertiary structure.

## Introduction

Humoral regulation of red blood cell production was first proposed at the beginning of this century (Carnot, P., *et al.*, *C.R. Seances Acad. Sci.* 143:432-35 (1906)). Convincing physiologic experiments documenting the existence of erythropoietin (Epo) (Krumdieck, N., *Proc. Soc. Exp. Biol. Med* 54:14-17 (1943); Reissmann, K.R., *Blood* 5:372-380 (1950); Erslev, A., *Blood* 8:349-357 (1953); Jacobson, L.O., *et al.*, *Nature* 179:633-634 (1957)) were followed by its purification (Miyake, T., *et al.*, *J. Biol. Chem.* 252:5558-5564 (1977)) and partial structural characterization (Lai, P.-H., *et al.*, *J. Biol. Chem.* 261:3116-3121 (1986)). The molecular cloning of this biologically and clinically important cytokine (Jacobs, K., *et al.*, *Nature* 313:806-810 (1985); Lin, F.-K., *et al.*, *Proc. Nat. Acad. Sci. USA* 82:7580-7585 (1985)) has led to further understanding of its properties (Sasaki, H., *et al.*, *J. Biol. Chem.* 262:12059-12076 (1987); Davis, J.M., *et al.*, *Biochemistry* 26:2633-2638 (1987)).

The binding of Epo to its cognate receptor (D'Andrea, A.D., *et al.*, *Cell* 57:277-285 (1989)) on erythroid progenitors in the bone marrow results in salvaging these cells from apoptosis (Koury, M.J., *et al.*, *Science* 248:378-381 (1990)), allowing them to proliferate and differentiate into circulating erythrocytes. The Epo receptor is a member of an ever enlarging family of cytokine receptors (Bazan, F., *Biochem. Biophys. Res. Commun.* 164:788-796 (1989)). In like manner, Epo shares weak sequence homology with other members of a family of cytokines which also include growth hormone, prolactin, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, GCSF, GM-CSF, M-CSF, oncostatin M, leukemia inhibitory factor and ciliary neurotrophic factor (Parry, D.A.D., *et al.*, *J. Mol. Recognition* 1:107-110 (1988); Bazan, F., *Immunology Today* 11:350-354 (1990); Manavalan, P., *et al.*, *J. Protein Chem.* 11:321-331 (1992)). The genes encoding these proteins have similar numbers of exons as well as a clear relationship between intron-exon boundaries and predicted  $\alpha$ -helical structure. These similarities

have led to the prediction that this family of cytokines share a common pattern of folding into a compact globular structure consisting of four amphipathic  $\alpha$ -helical bundles. Such theoretical models of the structures of human growth hormone (Cohen, F.E., et al., *Proteins: Struct. Func. Genet.* 2:162-166 (1987)) and IL-4 (Curtis, B.M., et al., *Proteins: Struct. Func. Genet.* 11:111-119 (1991)) have been in remarkably good agreement with subsequent structures established by X-ray diffraction (HGH) (Abdel-Meguid, S.S., et al., *Proc. Natl. Acad. Sci. USA* 84:6434-6437 (1987); deVos, A.M., et al., *Science* 255:306-312 (1992)) or by multidimensional NMR (IL-4) (Redfield, C., et al., *Biochemistry* 30:11029-11035 (1991); Powers, R., et al., *Science* 256:1673-1677 (1992)). Moreover, the crystal structures of GM-CSF (Diederichs, K., et al., *Science* 254:1779-1782 (1991)) and monomeric M-CSF (Pandit, J., et al., *Science* 258:1358-1362 (1992)) are also in reasonable agreement with their predicted structures.

Thus far, the structure of Epo has not been analyzed by either X-ray diffraction or by NMR. In order to begin to gain an understanding of structure-function relationships, we have taken a three-pronged approach:

- a) Sequence determination of Epo from mammals of different orders in order to establish regions of homology. This work is described in Example I.
- b) Construction of a model of the three-dimensional structure of Epo, followed by the design and preparation of muteins that test this model. These experiments are presented in this Example.
- c) Design and testing of muteins that provide information on receptor binding domain(s). This work is presented in Example III.

## Materials and Methods

### *Computer-based Modeling of Structure*

*Prediction of Secondary Structure.* Epo sequences from human, monkey, mouse, rat, sheep, pig, and cat were aligned (See Example I), and 5 examined using a hierarchical approach to secondary structure prediction that assumes that these proteins are members of the  $\alpha/\alpha$  folding class (Levitt, M. et al., *Nature* 261:552-558 (1976)). First, the pattern-based method of Cohen, F.E. et al., *Biochemistry* 25:266-275 (1986) for turn prediction was used to delimit sequence blocks likely to contain secondary structure. Predictions using 10 the methods of Garnier, J. et al., *J. Mol. Biol.* 120:97-120 (1978) and Chou, P.Y. et al., *Ann. Rev. Biochem.* 47:251-276 (1978) suggested  $\alpha$ -helical regions within these blocks. Finally, helical wheel projections were used to examine and then limit helix length based on preserving amphipathic character 15 as codified in the work of Presnell, S.R. et al., *Biochemistry* 31:983-993 (1992). The locations of glycosylation sites were also used to suggest helix boundaries.

*Tertiary Structure Prediction.* Earlier investigations have revealed the general principles of helix-to-helix packing in globular proteins (Richmond, T.J., et al., *J. Mol. Biol.* 119:537-55 (1978)). Exploring these principles, 20 Cohen, F.E. et al., *J. Mol. Biol.* 132:275-288 (1979) developed a method for the generation of three-dimensional protein structures from the secondary structure assignment. These methods have been applied to myoglobin, tobacco mosaic virus coat protein, growth hormone,  $\alpha$ - and  $\beta$ -interferon, IL-2, and IL-4 (Cohen, F.E., et al., *J. Mol. Biol.* 132:275-288 (1979); Cohen, F.E., et al., 25 *Science* 234:349-352 (1986); Cohen, F.E., et al., *Proteins: Struc. Func. Gen.* 2:162-166 (1987); Sternberg, M.J.E., et al., *Int. J. Biological Macromolecules* 4:137-144 (1982)).

The algorithm for tertiary structure generation is divided into four computations. The program **aapatch** identifies clusters of hydrophobic residues within the putative helices that could mediate helix-helix interactions (Richmond, T.J., *et al.*, *J. Mol. Biol.* 119:537-55 (1978)). **Aafold** generates  
5 all possible helix pairings according to the location and geometric preferences of the interaction sites. **Aabuild** generates the three-dimensional models of all possible structures from the list of helix pairings (from **aafold**) and subject to stearic restrictions and geometric constraints on chain folding. In the final  
10 step, **aavector** applies the user defined distance constraints (e.g., disulfide bridges) to the structures generated. At this stage, coordinates have been specified only for residues in the core  $\alpha$ -helices. For residues in sequentially distinct loops, lower bounds on the inter-residue distances can be inferred from the relevant helix terminus.

#### *Preparation of Epo Muteins*

15         *Construction of the Mutagenic/Mammalian Expression Plasmid.* A M13 plasmid, containing a 1.4 Kb EcoRI-EcoRI human Epo cDNA insert ( $\lambda$ HEpo FL12) was a gift from Genetics Institute (Cambridge, MA) (Jacobs, K., *et al.*, *Nature* 313:806-810 (1985)). A 943 bp EcoRI-BglII fragment, corresponding to the complete coding sequence of the wild type human  
20 erythropoietin, including untranslated regions 216 bp upstream and 183 bp downstream, was inserted into the mammalian expression plasmid pSG5 (Stratagene) (Sambrook, J., *et al.*, In "Molecular Cloning: A Laboratory Manual", 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp. 1.38-1.41 (1989)) and named pSG5-Epo/WT.  
25         *Site-Directed Mutagenesis* was carried out according to the protocol described by Kunkel, T.A., *et al.*, *Meth. Enzymol.* 154:367-382 (1987). Single-stranded DNA was rescued from the pSG5-Epo/WT phagemid grown overnight in *E. coli* CJ236, in 2XYT media containing M13KO7 helper phage

(In Vitrogen) and 70 µg/ml kanamycin (Sigma). The resulting uracil-containing ssDNA was used as a template for mutagenesis. Oligonucleotides (24 to 46 mer) were synthesized with their 5' and 3' ends complementary to the target wild type Epo sequence. A large variety of mutations (base substitutions, deletions and insertions) were created at the centers of the mutagenic primer sequences. Annealing of the phosphorylated primers (10:1 oligonucleotide/DNA template molecular ratio) was performed in 10 µl of a 20 mM Tris-HCl pH 7.4, 2 mM MgCl<sub>2</sub>, 50 mM NaCl solution. The reactions were incubated at 80°C for 5 minutes, then allowed to cool slowly to room temperature over a one hour period. The DNA polymerization was initiated by the addition as a mix of 1 µl of 10X synthesis buffer (100 mM Tris-HCL pH 7.4, 50 mM MgCl<sub>2</sub>, 10 mM ATP, 5 mM each dNTPs, 20 mM DTT), 0.5 µl (8 units) of T4 DNA ligase and 1 µl (1 unit) of T4 DNA polymerase (Boehringer Mannheim). After 2 hours at 37°C, 80 µl of 1X TE was added. 5 µl of the diluted reaction mix was used to transform competent *E. coli* NM522 (ung<sup>+</sup>, dut<sup>+</sup>).

Since a 40-80% mutation yield is normally obtained, 4 to 5 double-stranded plasmid clones from each reaction were sequenced with 7-deaza-dGTP and Sequenase (U.S. Biochemical) (Sanger, F., et al., *Proc. Natl. Acad. Sci. USA* 74:5463-5467 (1977)). As a rule, the entire coding sequences of the Epo mutants were examined for the presence of unwanted mutation by sequencing or restriction enzyme mapping.

*Production of Wild Type Epo and Epo Mutants in Mammalian Cells.*

Cos7 cells grown to approx. 70% confluence were transfected with 10 µg recombinant plasmid DNA per 10 cm dish using the calcium phosphate precipitation protocol (Kingston, R.E., et al., In "Current Protocols in Molecular Biology", Green Publishing Associates & Wiley Interscience, NY, pp. 9.1.1-9.1.9 (1991)). As a control of transfection efficiency, in several experiments 2 µg of pCH110 plasmid (Pharmacia) was co-transfected and β-galactosidase activity measured in the cytoplasmic extracts.

*RNA Blot-Hybridization Analysis.* Total RNAs were prepared from cultured Cos7 cells (Chirgwin, J.M., et al., *Biochemistry* 18:5294-5299 (1979)) and 2 µg samples electrophoresed on a 1.1% agarose gel containing 2.2 M formaldehyde. Transfer to GeneScreen Plus filters (New England Nuclear) and hybridization with a <sup>32</sup>P-labeled wt Epo probe were carried out as previously described (Faquin, W.C., et al., *Blood* 79:1987-1994 (1992)).

5           *Quantitation of Transiently-Expressed Recombinant Epos.* The amount of secreted protein in the supernatants of transfected Cos7 was determined by a radioimmune assay (RIA). The RIA was performed using a  
10          high titer rabbit polyclonal antiserum raised against the human wild type Epo and produced in our laboratory. <sup>125</sup>I-labeled recombinant Epo was obtained from Amersham. Details of the protocol have been published elsewhere (Faquin, W.C., et al., *Exp. Hematol.* 21: 420-426 (1993)).

15          *Immunoprecipitation of <sup>35</sup>S-labeled Epo Proteins.* Three days after transfection, the Cos7 monolayers were washed extensively with 1X PBS and the cells incubated for 20 minutes at 37°C in 2 ml of Met/Cys Minimum Essential Media Eagle modified. In each culture dish, 100 µl of TRAN35S-LABEL ([<sup>35</sup>S]-cysteine and [<sup>35</sup>S]-methionine, ~10 mCi/ml, ICN Biochemical) was then added. After two hours, the conditioned media were harvested and 20          cellular extracts were prepared by lysis in radioimmune precipitation buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.02% (w/v) sodium azide, 0.1% (w/v) SDS, 0.5% (w/v) sodium deoxycholate, 1% (w/v) Triton X-100, 1 mM phenylmethanesulfonyl fluoride and 1 µg/ml aprotinin). Samples were 25          precleared with rabbit preimmune serum/protein A-Sepharose CL-4B (Pharmacia) for two hours. Immunoprecipitations were performed overnight with our polyclonal antibody specific for human recombinant wild type Epo and immunoabsorbed with protein A-Sepharose CL-4B. Immunoprecipitates were run on 15% SDS-polyacrylamide gels (Laemmli, U.K., *Nature* 227:680-685 (1970)) and analyzed by autoradiography after treatment with 30          Enhance (New England Nuclear).

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**Bioassays.** The dose-dependent proliferation activities of wild type Epo and Epo muteins were assayed *in vitro* using three different target cells: murine spleen cells, following a modification of the method of Krystal (Krystal, G., *Exp. Hematol.* 11:649-60 (1983); Goldberg, M.A., *et al.*, *Proc.*

- 5 *Natl. Acad. Sci. USA* 84:7972-7976 (1987)); Epo responsive murine erythroleukemia cell line, developed by Hankins (Hankins, W.D., *et al.*, *Blood* 70:173a (1987)); and human Epo-dependent UT-7/Epo cell line, derived from the bone marrow of a patient with acute megakaryoblastic leukemia (Komatsu, N., *et al.*, *Cancer Res.* 51:341-348 (1991)). After 22 to 72 hours  
10 of incubation with increasing amounts of recombinant proteins, cellular growth was determined by [<sup>3</sup>H]-thymidine (New England Nuclear) uptake or by the colorimetric MTT assay (Sigma) (Yoshimura, A., *et al.*, *Proc. Natl. Acad. Sci. USA* 87:4139-4143 (1990)).

**Bacterial Expression.** The wild type Epo target, corresponding to the  
15 nucleotide sequence coding for the mature protein, was PCR-amplified using appropriate primers. In the sense primer an NdeI site (CATATG) was placed immediately 5' to the Ala<sub>1</sub> codon of the mature protein. In the antisense primer a BglII site was placed 3' to the TGA stop codon. After enzymatic digestion, the 516 bp PCR fragment was inserted in an NdeI/BamHI-cut  
20 pET16b plasmid (Novagen), which has a T7 promoter followed immediately by the lac operator. IPTG induction of transformed *E. coli* BL21 (DE3) (T7 RNA polymerase<sup>+</sup>, *lon*<sup>+</sup>, *ompT*<sup>+</sup>) resulted in high levels of expression of a fusion protein with a ten histidine stretch at the amino terminus. The oligo-His tag allowed the binding of the produced (His<sub>10</sub>)-Epo on a nickel affinity  
25 resin and its elution by increasing imidazole concentrations in presence of phenylmethylsulfonyl fluoride (Sigma). Most of the produced protein formed insoluble aggregates and was solubilized and affinity-purified under denaturing conditions in 6 M guanidine-HCl. Oxidative refolding was performed by overnight dialysis at 4°C against 50 mM Tris-HCl pH 8.0, 40 μM CuSO<sub>4</sub> and  
30 2% (wt/vol) Sarkosyl. Soluble protein was further dialyzed against 20 mM Tris-HCl pH 8.0, 100 mM NaCl, 2 mM CaCl<sub>2</sub> and subjected to factor Xa

(New England Biolabs) cleavage to remove the NH<sub>2</sub>-polyHis sequence. Monitoring of the fusion protein following induction and during the various steps of purification was done by electrophoresis on a 15% polyacrylamide-SDS gel, stained with Coomassie Brilliant Blue. Alternatively, the His-Epo fusion protein was detected on Western Blot (Gershoni, J.M., *et al.*, *Anal. Biochem.* 131:1-15 (1983)), using a 1/2000 dilution of our wt native Epo polyclonal antibody and a second biotinylated rabbit-specific antibody which is detected with a streptavidin-alkaline phosphatase conjugate (Amersham).

*In Vitro Transcription/Translation.* Sense and antisense primers, creating new BglII sites respectively 5' and 3' of the initiator and stop codons, were used in a polymerase chain reaction on pSG5-Epo/WT template. After BglII cleavage, the 594 bp PCR-fragment was subcloned into p SP64T (Krieg, P.A., *et al.*, *Nucl. Acid. Res.* 12:7057-7070 (1984)). This SP6 containing vector provides 5' and 3' flanking regions from Xenopus  $\beta$ -globin mRNA, which allow efficient *in vitro* transcription/translation. Previous experiments showed poor yields of *in vitro* translated protein, when using the GC-rich natural 5' Epo untranslated region (UTR). One step *in vitro* transcription/translation was carried out by incubation of 1  $\mu$ g of circular p64T-Epo in a 50  $\mu$ l reaction volume of SP6-TnT coupled rabbit reticulocyte lysate system (Promega), in presence of [<sup>35</sup>S]-cysteine (1200 Ci/mM, New England Nuclear). In some cases, canine pancreatic microsomal membranes were added to the reaction mix. A purified GST-human Epo receptor extracellular domain fusion protein (EREx) was a gift from W. Harris and J. Winkelman, and the binding of the <sup>35</sup>S-labeled translation products onto EREx-glutathione agarose beads were performed as described (Harris, K.W., *et al.*, *J. Biol. Chem.* 267:15205-15209 (1992)).

## Results

### *Construction of a Model of the Three-Dimensional Structure of Erythropoietin*

From an analysis of the putative Epo helix sequences, aapatch 5 identified eight possible helix-helix interaction sites. In principle, these sites could be used to generate  $1.6 \times 10^4$  structures. Of these, only 706 maintained the connectivity of the chain and were sterically sensible. These structures resembled four helix bundles, an increasingly common motif in protein structure (Presnell, S.R., et al., *Proc. Natl. Acad. Sci.* 86:6592-6596 (1989)).

10 The structures that were not compatible with the native disulfide bridge between Cys<sub>7</sub> and Cys<sub>161</sub> were eliminated. This reduced the total number of structures from 706 to 184 (total computer time approximately 1 hr. on a Silicon Graphics IRIS 4D/35G). The remaining structures were then rank ordered by solvent-accessible surface contact area, a measure of the validity 15 of model structures. The most compact structures were right handed, all anti-parallel four-helix bundles with no overhand connections, but this may be an artifact of a failure to add the polypeptide chain that forms the loops to the helical core constructed by aabuild. The other less compact structures were left-handed four-helix bundles with two overhand loops, a topology previously seen in the structures of IL-4 and growth hormone. We suspect that this is the likely structure for Epo. The consensus for assignments of putative  $\alpha$ -helices 20 in human Epo are summarized in Table III. First, analysis of the topological distribution of known four-helix bundle structures indicate that nearly all examples have an antiparallel orientation (Presnell, S.R., et al., *Proc. Natl. Acad. Sci.* 86:6592-6596 (1989)). Second, the left-handed four-helix bundles 25 with two overhand connections arranges the four amphipathic helices to form a compact hydrophobic core. Finally, a path for the loop regions of Epo can be imagined by analogy to IL-4 and growth hormone that preserves the compact globular nature of the Epo model structure. Figure 9 shows

schematic representations of the possible topological interactions between the four-anti-parallel  $\alpha$ -helical bundles.

Table III

Predicted $\alpha$ -helical regions of the mature erythropoietin protein				
	Helix	N-terminus	C-terminus	Potential Helix-Helix Interaction Sites
5	A	9	22	19
	B	59	76	63, 67, 70, 71
	C	90	107	95, 102
	D	132	152	141
10	Data were obtained using the various algorithms for secondary and tertiary structure generations described under Materials and Methods.			

Several authors have suggested that the helical cytokines form a structural superfamily (Cohen, F.E., *et al.*, *Science* 234:349-352 (1986); Parry, D.A.D., *et al.*, *J. Mol. Recognition* 1:107-110 (1988); Bazan, J.F., *Biochem. Biophys. Res. Comm.* 164:788-795 (1989); Cosman, D., *et al.*, *Trends Biochem. Sci.* 15:265-270 (1990); Bazan, J.F., *Proc. Natl. Acad. Sci. U.S.A.* 87:6934-6938 (1990)). On the basis of both the mature protein and the individual  $\alpha$ -helices, Epo seems to be more closely related to growth hormone, prolactin, IL-6 and GM-CSF rather than the other members of the helical cytokine superfamily. Nevertheless, recent improvements in algorithms for the identification of distant evolutionary relationships between proteins from structural fingerprints suggested that it might be possible to align the IL-4 structure to the Epo sequences. The Eisenberg (Bowie, J.U., *et al.*, *Science* 253:164-170 (1991)) structural environment and 3D-1D profile methods are a powerful tool for recognizing that a sequence is compatible with a known structure, e.g., a four-helix bundle. The NMR structure of IL-4 from Smith, L.J., *et al.*, *J. Mol. Biol.* 224:899-904 (1992) was used to construct a 3D-1D profile. A mixture of sequences including four helix

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bundles, globins, and non-helical structures were aligned against the IL-4 profile. Not surprisingly, the IL-4 structures from human and mouse gave the highest scores ( $Z^2 = 22.8$  and  $8.1$ ). However, the other known four-helix bundle cytokines known to share a similar fold with IL-4, e.g., human growth  
5 hormone (Abdel-Meguid, S.S., *et al.*, *Proc. Natl. Acad. Sci.* **84**:6434-6437 (1987)) ( $Z = 2.3$ ) and GM-CSF (Diederichs, K., *et al.*, *Science* **254**:1779-1782 (1991)) ( $Z = 2.3$ ) fared no better than some globin sequences (Kuroda's and slug sea hare globin,  $Z = 5.0$  and  $4.8$ ) that adopt a distinct tertiary structure. The results for the human and sheep Epo sequences were  
10 also ambiguous ( $Z = 1.6$  and  $0.8$ ). These results suggest that while profile methods are a powerful tool for recognizing structural similarity, their failure to identify homology does not exclude the possibility that two proteins share a common fold. For distantly related or unrelated structures, current profile methods cannot replace *de novo* methods for tertiary structure prediction.

15     *Design and Expression of Epo Muteins that Test the Proposed Structure*

To test the proposed four  $\alpha$ -helical bundle structure of erythropoietin and at the same time to attempt to locate functional domains, we created by site-directed mutagenesis a series of deletion, insertion and replacement mutants. These muteins were designed to analyze the principal predicted  
20 structural features of the molecule:  $\alpha$ -helices, interconnecting loops, as well as the NH<sub>2</sub> and COOH termini. Structural and functional implications of the disulfide bridges and the glycosylation sites were also investigated.

*$\alpha$  Helices.* Short amino acid deletions were prepared in, or close to, the predicted A, B, C and D  $\alpha$  helices. Human wild type and muteins were

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25     <sup>2</sup>Z-scores are used to describe the normalized weight associated with a profile score. A distribution is built from a collection of sequences with a mean 2 score of 0.0 and a standard deviation of 1.0. Z-scores greater than 6.0 are associated with significant alignments. Z-scores between 3.0 and 6.0 may or may not be structurally relevant.

transiently expressed in Cos7 cells. Northern blot analyses demonstrated that all the mutant plasmids produced about the same amount of mRNA as that of the wild type (data not shown). Yet, no detectable amount of Epo protein could be found in the Cos7 supernatants, either by radioimmunoassay or by 5 bioassay using various Epo-dependent cell lines. Table IV summarizes these findings.

TABLE IV  
Short deletions in, or close to,  $\alpha$  helices

mutants.

$\Delta$ 12-16	9 ↓ 22	A HELIX	<ul style="list-style-type: none"> <li>- RNA levels comparable to WT.</li> <li>- no detectable Epo in the Cos7 supernatant, both by RIA and bioassay.</li> </ul>
$\Delta$ 65-69	59 ↓ 76	B HELIX	
$\Delta$ 96-100	90	C HELIX	
$\Delta$ 105-109	↓ 107		
$\Delta$ 122-126			
$\Delta$ 131-135	132	D HELIX	
$\Delta$ 140-144			
$\Delta$ 142-150			
$\Delta$ 152-155	152		
$\Delta$ 156-160			

Predicted N and C termini of each  $\alpha$  helix are indicated in the vertical boxes.

An example of SDS-PAGE of immunoprecipitants from *in vivo*  $^{35}$ S labeling is presented in Figure 10. As expected, when Cos7 cells were transfected with pSG5-Epo/WT, a 35-37 KD band was detected in the supernatant. In contrast, the deletion mutants (Table IV) could be detected in 5 cellular extracts but were not exported from the cells. Figure 10 shows the

cytoplasmic retention of the mutein  $\Delta$ 140-144, lacking four residues in the middle of the predicted D helix. The apparent molecular weight (around 28 kD) is less than expected for a five-amino acid deletion. Therefore not only the secretion, but also the glycosylation, seem to be impaired. None of these 5 muteins had deletion of glycosylation sites. It is likely that full glycosylation of Epo requires conservation of its molecular architecture. Similar results (reported in Table IV) were obtained for all the muteins having partial deletion of an  $\alpha$  helical peptide segment.

Because contaminants in crude Cos7 cellular extracts severely interfere 10 with the radioimmunoassay, no direct Epo quantitation was possible. However, aliquots of hypotonic extracts of Cos7 transfected with wild type Epo were able to sustain HCD57 proliferation. No similar biological activity was found for muteins with limited deletion of a helices.

*Interconnecting Loops.* The peptide segment joining A and B helices 15 presents several interesting features (Figure 11). AB loop consists of 36 amino acids. Two N-glycosylation sites and a small disulfide bridge are located in the first half and their biological implications will be discussed later. The COOH end of the AB loop contains a stretch of amino acids that is strongly conserved among mammals (See Example I). Alignments of human, 20 monkeys, cat, mouse, rat, pig, and sheep Epos showed a consensus sequence: DTKVNFYAWKR(M/I)(E/D)VG (residues 43 to 57) [SEQ. ID NO: 4]. Three deletions were constructed:  $\Delta$ 43-47,  $\Delta$ 48-52, and  $\Delta$ 53-57, and 25 transiently expressed in Cos7. The amount of muteins detected by RIA in the supernatants of transfected cells was 10 to 40% lower than observed with wild type Epo (Figure 11A). Nevertheless, the three secreted muteins were biologically active. However, because  $\Delta$ 48-52 exhibited a marked decrease of the specific bioactivity, this site was studied in more detail by means of serine replacements. Krystal *ex vivo* bioassay as well as HCD57 and 30 UT-7/Epo *in vitro* bioassays showed that these Ser mutants had biological activities similar to that of wild type (Figure 11B). Therefore, the observed decreases in both RIA and bioassay for the three deletion mutants are likely

to be the result of changes of structural conformation. The long length of loop AB may be critical for the up-up-down-down topography. A shorter AB segment may impose a strain on the interhelical connection. Chou and Fasman algorithms (Chou, P.Y., *et al.*, *Ann. Rev. Biochem.* 47:251-276 (1978)) predicted a short  $\beta$ -sheet structure from residues 44 to 51 ( $\langle P_a \rangle$ ;  $\langle P_b \rangle$ , 1.005 < 1.196). The presence of a short region of  $\beta$ -sheet in the connection between helices 1 and 2 (A and B) have been documented in the analyses of the three-dimensional structures of IL-4 (Redfield, C., *et al.*, *Biochemistry* 30:11029-11045 (1991); Powers, R., *et al.*, *Science* 256:1673-1677 (1992)), GM-CSF (Diederichs, K., *et al.*, *Science* 254:1779-1782 (1991)), and monomeric M-CSF (Pandit, J., *et al.*, *Science* 258:1358-1362 (1992)). In contrast, in human GH a short segment of a helix is found at the same location (Abdel-Meguid, S.S., *et al.*, *Proc. Natl. Acad. Sci. USA* 84:6434-6437 (1987)). The structure/function implications of these short features are not yet understood.

Helix B is linked to helix C by a much shorter segment (residues 77 to 89) and contains in its center the third N-glycosylation site (Asn83). When the  $\Delta$ 78-82 mutein was expressed, a secreted protein was detected in the conditioned medium and conferred proliferative bioactivity on Epo-dependent cell lines (see Figure 16).

A similar long crossover connection (23 amino acids) is found between helix C and helix D. In contrast to what we previously observed for loop AB, a large deletion of nine residues at position 111-119 or a seven amino acid insertion of a myc epitope after residue 116 did not affect the secretion of these muteins (Figure 12). Furthermore, these two proteins had normal specific activity, as seen by the ratio of bioassay to RIA. Our rabbit polyclonal antibody raised against the native form of the human wild type fully recognized the two mutants, demonstrating that the overall spatial conformation of Epo was well preserved. According to the algorithm of Emini, E.A., *et al.*, *J. Virol.* 55:836-839 (1985), the residues 111-119 are predicted to be at the surface of the molecule. Primary amino acid alignments

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of mammalian Epo showed a large variation in the sequence of residues 116 to 130, including amino acid deletion, insertion, and substitution (See Example I). Surprisingly, when the deletion  $\Delta$ 122-126 mutein, which removed the O-glycosylation site (Ser 126), was transiently expressed in monkey cells, protein secretion was inhibited. Both rodents, rat and mouse, lack the O-glycosylation site because of a Ser126 to Pro replacement. Furthermore, when a Ser126 replacement mutein was expressed in normal CHO cells, (DeLorme, E., et al., *Biochemistry* 33:9871-9876 (1992)) or when wild type Epo was expressed in cells having a defect in O-linked glycosylation (Wasley, L.C., et al., *Blood* 77:2624-2632 (1991)), neither secretion nor biological activity were impaired. Therefore, failure of secretion of the  $\Delta$ 122-126 mutein may be the result of some other structural alteration. In particular, the proline residue at position 122 is invariant among mammals.

*N and C Termini.* Deletion of residues 2 to 6 only slightly affected the processing of a biologically active protein (see Figure 16). This deletion may impair cleavage of the propeptide, therefore explaining the lower yield of secreted Epo mutein in comparison to that of wild type. The fact that the mature monkey protein has an elongated (Val-Pro-Gly) N-terminus strongly suggests that the N-terminal part is not involved in the bioactivity of the molecule. Further evidence comes from the results, reported below, on the N-poly-His-Epo fusion protein expressed in *E. coli*, and also from the identical binding of *in vitro* translated  $^{35}$ S-labeled wild type Epo onto EREX-glutathione agarose beads (Figure 13), with or without addition of canine pancreatic microsomal membranes which permit cleavage of the propeptide.<sup>3</sup>

The C-terminal sequence following helix D can clearly be divided into two distinct domains, separated by Cys161. The residues 151 to 161 were of special interest because they are highly conserved among mammals (See Example I). There are only two substitutions: Lys164 replaced by a Thr in

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<sup>3</sup>All the mutants described in this example were subcloned into pSPG4T plasmid.

artiodactyls and cat, and Ala160 is replaced by a Val in mouse Epo. Both the Δ152-155 and the Δ156-160 muteins remained in the cytosol of the transfected Cos7 (Table IV). One possible explanation is that the residues 152 to 160 may, in fact, participate in the D helix. We predict that Gly151 is the break 5 point of the structure. However, it is possible that this residue causes only a bend in the a helical structure and helix D may extend to Gly158.

The C-terminal part of the protein (residues 162 to 166) is clearly not involved in any structural or functional feature. Thus, the deletion of the four last amino acids or the replacement of residues 162-166 by either a KDEL 10 sequence or a poly-histidine sequence<sup>4</sup> did not modify the specific activity of the erythropoietin (Figure 14). Radioimmunoassay revealed that the secretion of the KDEL-tail mutein in the media of transfected cells was 45% less than normally obtained with the wild type Epo. However, when compared to the wild type, this mutein had more biological activity in the hypotonic Cos7 cell 15 extracts. The KDEL COOH-terminal sequence has been shown to be essential for the retention of several proteins in the lumen of the endoplasmic reticulum (Andres, D.A., et al., *J. Biol. Chem.* 266:14288-14282 (1991)). Nevertheless, because of overproduction in transiently expressed cells, a large percentage of recombinant protein escaped into the media.

20        *Disulfide Bridges.* Wang et al. demonstrated that the biological activity of Epo was lost irreversibly if the sulfhydryl groups were alkylated (Wang, F.F., et al., *Endocrinology* 116:2286-2292 (1985)). The mature human Epo has two internal disulfide bonds: Cys7=Cys161, linking the NH<sub>2</sub> and COOH termini of the protein and a small bridge between Cys29 and 25 Cys33. Cys33 was previously changed to Pro by site-directed mutagenesis, and the resulting protein was reported to have greatly reduced *in vitro* biological activity (Lin, F.-K., *Molecular and Cellular Aspects of*

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30        <sup>4</sup>The poly-His tail wild type mutant was purified by means of nickel affinity chromatography which will enable quantitation of cytosolic-retained mutants. The (His)<sub>6</sub> COOH-terminal sequence has been appended to all the muteins described in this example.

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*Erythropoietin and Erythropoiesis H8:23-36*, NATO AS1 Series, Springer-Verlag, Berlin Heidelberg (1987)). However, rat and mouse Epos have the same substitution and yet exhibit full cross-species bioactivity. To resolve the role of the small disulfide bridge in human Epo function, we created a 5 C29Y/C33Y double mutation. The resulting mutein was normally processed and showed the same *in vitro* bioactivity as the wild type (Figure 11A).<sup>5</sup> Furthermore, the deletion of five amino acid residues (Δ32-36) did not impair the secretion of a biologically active mutein. These data demonstrated that only the native and fully conserved disulfide bridge Cys7=Cys161 is crucial for the 10 preservation of the molecular structure of erythropoietin.

*Functional Role of the Glycosylation.* Natural or recombinant human Epo is a heavily glycosylated protein; 40% of its molecular weight is sugars (Davis, J.M., et al., *Biochemistry* 26:2633-2638 (1987)). The protein has three N-linked oligosaccharide chains, located at amino acid positions 24 15 and 38 (in predicted loop AB) and position 83 (in loop BC). It has one O-linked carbohydrate chain at position 126 (in loop CD), which is missing in rodents. The role of these sugar chains in the biological activity of the human hormone has been extensively studied. Site-directed mutagenesis at the N-glycosylation sites demonstrated that even though the sugars were important 20 for proper biosynthesis and secretion, their removal did not affect *in vitro* activity. This finding was corroborated by several investigators (Yamaguchi, K., et al., *J. Biol. Chem.* 266:20434-20439 (1991); Dubé, S., et al., *J. Biol. Chem.* 263:17516-17526 (1988)). However, Takeuchi, M., et al., *J. Biol. Chem.* 265:12127-12130 (1990) found that N-glycanase digestion results in 25 almost complete loss of biological activity. In contrast, there is general consensus that glycosylation plays a key role in the biological activity of the hormone *in vivo*. Various reports have demonstrated that the N-linked sugar chains enhance the stability and survival of Epo in the blood stream (Fukuda,

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30 <sup>5</sup>Two single replacement muteins (C29Y and C33Y) were also stable and had full biological activity (results not shown).

M.N., *et al.*, *Blood* 73:84-89 (1989); Tsuda, E., *et al.*, *Eur. J. Biochem.* 188:405-411 (1990)) and protect the hormone against clearance by the liver (Sasaki, H., *et al.*, *J. Biol. Chem.* 262:12059-12076 (1987)), thereby enabling the transit of the hormone from its site of production in the kidney to its target 5 cells in the bone marrow (Spivak, J.L., *et al.*, *Blood* 73:90-99 (1989)).

We expressed the wild type Epo in *E. coli*. Accordingly, the produced protein completely lacks sugar. The pET expression system was used and is detailed in the Methods section. IPTG induction of transformed 8L21 (DE3) bacterial strain rapidly results in a high level of expression of the poly-His Epo 10 fusion protein (Figure 15A and 15B). After three hours of induction, we obtained a typical yield of ~1 mg of total protein per ml of culture. However, the vast majority of the expressed protein was present in the inclusion bodies and therefore its solubilization and purification on the nickel beads was performed in 6 M guanidine-HCl. Oxidative refolding and factor 15 Xa cleavage resulted in soluble forms (Figure 15C) and the *in vitro* biological activity was tested on HCD57 cells. The cleaved *E. coli* recombinant Epo showed a notable decrease of the specific activity (10% less than the fully glycosylated mammalian expressed protein), but was still able to maintain HCD57 proliferation. The observed reduction of *in vitro* activity is likely to 20 be due to improper refolding of the insoluble protein and impaired physical stability of the *E. coli* Epo as previously reported (Narhi, L.O., *et al.*, *J. Biol. Chem.* 266:23022-23026 (1991)). However, the fact that the *E. coli* Epo was still able to trigger HCD57 growth indicated an overall preservation of the molecular structure. The uncleaved fusion protein, with 10 His residues at the 25 N-terminus, exhibited a 67% loss in biological activity when compared to the cleaved protein. Thus, the addition of a 20-residue sequence to the N-terminus partially inhibited the biological activity.

## Discussion

Currently the accrual rate of new protein sequences through gene cloning far outstrips the rate of determination of three dimensional structure.

Epo is among a large number of biologically important proteins which have not yet been analyzed by X-ray diffraction or NMR. The problem is simplified by cumulative evidence that the structures of most proteins are likely to be variations on existing themes (Levitt, M., *et al.*, *Nature* 261:552-558 (1976)).

Indeed, as mentioned above, Epo appears to share common structural features with a large group of cytokines (Parry, D.A.D., *et al.*, *J. Mol. Recognition* 1:107-110 (1988); Bazan, F., *Immunology Today* 11:350-354 (1990); Manavalan, P., *et al.*, *J. Protein Chem.* 11:321-331 (1992)).

Computer-based prediction of structure can be reduced to a three stage process: secondary structure is predicted from the primary amino acid sequence and, when available, optical measurements. Analysis of Epo by circular dichroism reveals about 50%  $\alpha$  helix and no detectable  $\beta$  sheet (Lai, P.-H., *et al.*, *J. Biol. Chem.* 261:3116-3121 (1986); Davis, J.M., *et al.*, *Biochemistry* 26:2633-2638 (1987)). With the knowledge of disulfide bonds, secondary structural elements are then packed into a set of alternative tertiary structures.

The number of plausible arrangements can be reduced by empirical knowledge of preferred helix-helix packing geometries and the need for globular structure to form a hydrophobic core. The putative tertiary structure is then refined by standard force field calculations. Since there are a large number of alternate tertiary structures, the availability of experimentally determined structure of a homologous protein is critically

important. Thus the predicted model of Epo structure gains considerable validity by knowledge of the structures of GH (Abdel-Meguid, S.S., *et al.*, *Proc. Natl. Acad. Sci. USA* 84:6434-6437 (1987); deVos, A.M., *et al.*, *Science* 255:306-312 (1992)) and IL4 (Redfield, C., *et al.*, *Biochemistry* 30:11029-11045 (1991); Powers, R., *et al.*,

*Science* 256:1673-1677 (1992)).

We have tested the predicted four anti-parallel  $\alpha$ -helical bundle structure by means of site-directed mutagenesis. Deletions within predicted  $\alpha$  helices would be expected to destabilize tertiary structure, whereas deletions or insertions in non-helical segments should be permitted unless they impose undue strain on the structure. For example, a deletion in an overhand inter-helical loop may result in insufficient length to connect the two helices. Results that we have obtained on muteins produced in mammalian (Cos7) cells are summarized in Figure 16. Our measurements of the quantities of processed mutein by RIA may underestimate the true amount of secreted Epo.

Even a small deletion or insertion can result in a conformational change that may lead to impaired binding by our polyclonal antibody, raised against native human Epo. Thus the values for specific activity (biologic activity/RIA) that we report must be regarded as approximations. This caveat notwithstanding, our mutagenesis results are in good agreement with our proposed four  $\alpha$ -helical model of erythropoietin. The proper folding of Epo into its native tertiary structure is necessary for stability and biological function. Muteins with short deletions inside predicted  $\alpha$ -helices were not processed and exhibited no biological activity. In contrast, when deletions were created in predicted interconnecting loops, secreted proteins were detected, to varying degrees, both by radioimmunoassay and bioassay. Furthermore, additions or deletions at the N- or C-termini did not markedly impair the secretion and the biological activity of the Epo protein. Moreover, mutations at Cys29 and Cys33 showed that the small disulfide loop is not critical for biological activity. In order to delineate Epo's functionally important residues involved in the direct binding onto the Epo receptor, we have prepared and tested a series of amino acid replacements on the surfaces of the predicted  $\alpha$  helices. These experiments are described in a Example III.

**EXAMPLE III****Erythropoietin Structure-Function Relationships:  
Muteins That Test Functionally Important Domains*****Introduction***

5       The Epo receptor is a member of an ever enlarging family of cytokine receptors (Bazan, F., *Biochem. Biophys. Res. Commun.* 164:788-796 (1989)). In like manner, Epo shares weak sequence homology with other members of a family of cytokines which also include growth hormone, prolactin, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, G-CSF, GM-CSF, M-CSF, oncostatin M, leukemia inhibitory factor and ciliary neurotrophic factor (Parry, D.A.D. et al., *J. Mol. Recognition* 1:107-110 (1988); Bazan F., *Immunology Today* 11:350-354 (1990); Manavalan, P. et al., *J. Protein Chem.* 11:321-331 (1992)). The genes encoding these proteins have similar numbers of exons as well as a clear relationship between intron-exon boundaries and predicted  $\alpha$ -helical bundles. Such theoretical models of the structures of human growth hormone (Cohen, F.E. et al., *Proteins: Struct. Func. Genet.* 2:162-166 (1987)) and IL-4 (Curtis, B.M. et al., *Proteins: Struct. Func. Genet.* 11:111-119 (1991)) have been in remarkably good agreement with subsequent structures established by X-ray diffraction (HGH) (Abdel-Meguid, S.S. et al., 10      *Proc. Natl. Acad. Sci. USA* 84:6434-6437 (1987); de Vos, A.M. et al., *Science* 255:306-312 (1992)) or by multidimensional NMR (IL-4) (Redfield, C. et al., *Biochemistry* 30:11029-11035 (1991); Powers, R. et al., *Science* 256:1673-1677 (1992)). Moreover, the crystal structures of GM-CSF (Diederichs, K. et al., *Science* 254:1779-1782 (1991)) and monomeric M-CSF 15      (Pandit, J. et al., *Science* 258:1358-1362 (1992)) are also in reasonable agreement with their predicted structures.

The structure of Epo has not yet been analyzed by either X-ray diffraction or by NMR. In order to begin to gain an understanding of structure-function relationships, we have taken a three-pronged approach:

- a) Sequence determination of Epo from mammals of different orders in order to establish regions of homology. This work is described in Example I.
- b) Construction of a model of the three-dimensional structure of Epo, followed by the design and preparation of muteins that test this model. These experiments are presented in Example II.
- c) Design and testing of muteins that provide information on receptor binding domain(s). This work is presented in this Example.

10

## MATERIALS AND METHODS

*Construction of the Mutagenic/Mammalian Expression Plasmid.* A M13 plasmid, containing a 1.4 Kb *EcoRI-EcoRI* human Epo cDNA insert ( $\lambda$ HEpo FL12) was a gift from Genetics Institute (Cambridge, MA) (Jacobs, K. et al., *Nature* 313:806-810 (1985)). A 943 bp *EcoRI-BgIII* fragment, corresponding to the complete coding sequence of the wild-type human erythropoietin, including untranslated regions 216 bp upstream and 183 bp downstream, was inserted into the mammalian expression plasmid pSG5 (Stratagene ) (Sambrook, J. et al., In "Molecular Cloning: A Laboratory manual", 2nd ed. Cold Spring Harbor, Laboratory, Cold Spring Harbor, N.Y., vol. 1, pp.1.38-1.41 (1989)) and named pSG5-Epo/WT.

*Site-Directed Mutagenesis* was carried out according to the protocol described by Kunkel, T.A. et al., *Meth. Enzymol.* 154:367-382 (1987). Single-stranded DNA was rescued from the pSG5-Epo/WT phagemid grown overnight in *E. coli* CJ236, in 2XYT media containing M13K07 helper phage (In Vitrogen) and 70  $\mu$ g/ml kanamycin (Sigma, St. Louis, Mo). The resulting uracil-containing ssDNA was used as a template for mutagenesis. Oligonucleotides (24 to 46 mer) were synthesized with their 5' and 3' ends complementary to the target wild-type Epo sequence. A large variety of mutations (base substitutions, deletions and insertions) were created at the

centers of the mutagenic primer sequences. Annealing of the phosphorylated primers (10:1 oligonucleotide/DNA template molecular ratio) was performed in 10  $\mu$ l of a 20 mM Tris-HCl pH 7.4, 2 mM MgCl<sub>2</sub>, 50 mM NaCl solution. The reactions were incubated at 80°C for 5 minutes, then allowed to cool

- 5 slowly to room temperature over a one hour period. The DNA polymerization was initiated by the addition as a mix of 1  $\mu$ l of 10X synthesis buffer (100 mM Tris-HCl pH 7.4 50, mM MgCl<sub>2</sub>, 10 mM ATP, 5 mM each dNTPs, 20 mM DTT), 0.5  $\mu$ l (8 units) of T4 DNA ligase and 1  $\mu$ l (1 unit) of T4 DNA polymerase (Boehringer Mannheim). After 2 hours at 37°C, 80  $\mu$ l of 1X TE  
10 was added. 5  $\mu$ l of the diluted reaction mix was used to transform competent *E. coli* NM522 (ung<sup>+</sup>, dut<sup>+</sup>).

- Since a 40-80% mutation yield is normally obtained, 4 to 5 double-stranded plasmid clones from each reaction were sequenced with 7-deaza-dGTP and Sequenase (U.S. Biochemical) (Sanger, F. et al., *Proc. Natl. Acad. Sci. USA* 74:5463-5467 (1977)). As a rule, the entire coding sequences of the Epo mutants were examined for the presence of unwanted mutation by sequencing or restriction enzyme mapping.

*Production of Wild-Type and Epo Muteins in Mammalian Cells.*

- Cos7 cells grown to approx. 70% confluence were transfected with 10  $\mu$ g recombinant plasmid DNA per 10 cm dish using the calcium phosphate precipitation protocol (Kingston, R.E. et al., In "Current Protocols in Molecular Biology", Green Publ. Assoc. & Wiley Interscience, N.Y., vol. 1, pp. 9.1.1-9.1.9 (1991)). As a control of transfection efficiency, in several experiments 2  $\mu$ g of pCH110 plasmid (Pharmacia) was co-transfected and  $\beta$ -galactosidase activity measured in the cytoplasmic extracts.

- RNA Blot-Hybridization Analysis.* Total RNAs were prepared from cultured Cos7 cells (Chirgwin, J.M. et al., *Biochemistry* 18:5294-5299 (1979)) and 2  $\mu$ g samples electrophoresed on a 1.1% agarose gel containing 2.2 M formaldehyde. Transfer to GeneScreen Plus filters (New England Nuclear)  
30 and hybridization with a <sup>32</sup>P-labeled wt Epo probe were carried out as previously described (Fauquin, W.C. et al., *Blood* 79:1987-1994 (1992)).

*Quantitation of Transiently-Expressed Recombinant Epos.* The amount of secreted protein in the supernatants of transfected Cos7 was determined by a radioimmune assay (RIA). The RIA was performed using a high titer rabbit polyclonal antiserum raised against the human wild-type Epo and produced in our laboratory. <sup>125</sup>I-labeled recombinant Epo was obtained from Amersham. Details of the protocol have been published elsewhere (Faquin, W.C. et al., *Exp. Hematol.* 21: 420-426 (1993)).

*Bioassays.* The dose-dependent proliferation activities of wt and Epo muteins were assayed *in vitro* using three different target cells: murine spleen cells, following a modification of the method of Krystal (Krystal, G., *Exp. Hematol.* 11:649-660 (1983); Goldberg, M.A. et al., *Proc. Natl. Acad. Sci. USA* 84:7972-7976 (1987)); murine Epo responsive murine cell line, developed by Hankins (Hankins, W.D. et al., *Blood* 70:173a (1987)); and human Epo-dependent UT-7/Epo cell line, derived from the bone marrow of 15 a patient with acute megakaryoblastic leukemia (Komatsu, N et al., *Cancer Res.* 51:341-348 (1991)). After 22 to 72 hours of incubation with increasing amounts of recombinant proteins, cellular growth was determined by [<sup>3</sup>H]-thymidine (New England Nuclear) uptake or by the colorimetric MTT assay (Sigma) (Yoshimura, A. et al., *Proc. natl. Acad. Sci. USA* 87:4139-4143 20 (1990)).

## RESULTS AND DISCUSSION

Our objective is to identify the functionally important domains on the Epo molecule by preparing muteins that have altered specific activity, i.e. significantly high or low biological activity per unit mass of protein. Such 25 muteins must satisfy the following criteria: efficient secretion by Cos7 cells; full recognition (near normal binding affinity) by the polyclonal anti-Epo antiserum that we use in our radio-immune assay; preservation of the overall folding and three dimensional structure. In order to meet these criteria, the muteins that we employ in this study have only single amino acid

replacements. A comprehensive amino acid replacement scan of Epo would of necessity require the preparation and testing of a minimum of 166 muteins. Such an undertaking would be prohibitively labor intensive and costly.

In order to devise a rational strategy to reduce the number of required  
5 replacements, we have taken advantage of information we have obtained employing scanning deletion muteins (See Example II). On the basis of its primary amino acid sequence and disulfide bonds Epo, in common with other members of the cytokine family is predicted to have a four antiparallel amphipathic  $\alpha$ -helical bundle structure. We have shown that deletions in non-helical regions at the N-terminus, the C-terminus and in loops between helices result in the formation of protein that is readily secreted from the cell and is biologically active. These regions can be ruled out as functionally important domains such as the sites involved in the binding of Epo to its receptor. These results suggest that the functionally important contact sites are likely to reside  
10 on the predicted  $\alpha$  helices. Furthermore, since the amphipathic helices are predicted to bind to one another along their hydrophobic surfaces, the biologically relevant contact sites are likely to be residues predicted to be on the external surfaces of the helices. These considerations greatly restrict the  
15 number of mutations needed to provide a comprehensive and informative body  
20 of data.

Because structure-function studies on growth hormone and IL-4 have indicated that Helices A and D are involved in receptor binding, most of our muteins have been directed to these regions.

Results on Helix A muteins are shown in Figure 17. The top panel  
25 shows a helical wheel projection. Replacement by alanine of the predicted external residues 13, 18, 20 and 21 have no significant impact on biological activity as determined by the relative incorporation of [<sup>3</sup>H]-thymidine into Epo dependent HCD57 cells. However, the replacement of Arg14 by Ala showed markedly impaired biological activity.

30 Figure 18, top panel, shows a helical wheel projection for Helix D. Nine of the nine predicted external residues were replaced by alanine.

Mutations at positions 136, 139 and 140 gave rise to proteins that were efficiently secreted and had full biological activity (Figure 18, bottom). In contrast Ala replacement of either Lys140, Arg143, Ser146, Asn147 or Lys154 resulted in muteins with significantly (3-fold) increased biological activity. Replacement of Asn147 with Ala resulted in the greatest increase in biological activity. (Figures 23-25).

Because the C-terminal boundary of Helix D is uncertain, we tested alanine replacements at positions 152, 154 and 156 which are predicted to be in the adjacent non-helical segment at the C-terminus. As shown in Figure 19, 10 the Ala replacement at Lys154 had slightly increased biological activity while the Ala replacement at Tyr156 had slightly decreased biological activity.

The experiments reported above all involve testing the function of human Epo muteins on the mouse Epo responsive HCD57 cell line. Figure 20 shows a comparative display of those muteins that appear to have higher 15 biological activity than wild-type Epo.

In order to assess the significance of these results we have also tested these muteins in a more physiologic system, mouse erythroid progenitors prepared from the spleens of animals challenged with phenylhydrazine. This bioassay obviates the possible confounding effects of a malignant clonal cell 20 line such as HCD57 which was derived from mouse erythroleukemia virus.

As shown in Figure 21, results with erythroid cells from mouse spleen were very similar to those obtained with HCD57 cells.

In addition we have tested these muteins in an Epo responsive human cell line UT-7/Epo, derived from a patient with acute leukemia. This cell line 25 provides the opportunity of examining the interaction of human muteins with the human Epo receptor. As shown of ordinary in Figure 22, these results closely parallel those with the two mouse cell bioassays.

It should be noted that in several of the dose response curves, at the highest concentrations of Epo tested, there is a significant drop-off in the 30 incorporation of [<sup>3</sup>H]-thymidine. This is due to the fact that high levels of biologically active Epo causes terminal maturation and cessation of division.

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Because of this possibly confounding phenomenon, it is important to have, as in the experiments that we report here, a full dose response curve for each mutein with an adequate number of data points.

Taken together our results show that a restricted number of residues on 5 Helix A and on Helix D are involved in Epo's biological activity. Further experiments are in progress to determine if the observed alterations in biological activity can be explained by parallel changes in the binding affinity to the Epo receptor. In addition, at the sites that we have identified as involved in Epo's biological activity, we plan to make a set of additional 10 replacement muteins in order to further refine the ligand-receptor interaction. We are particularly interested in developing muteins with maximally increased biological activity, e.g. by preparing compound or multiplex replacements, each of which confers optimal enhancement of biological activity. Such a "superEpo" could have improved utility in the therapy of anemias.

15

*Conclusions*

We have produced a number of erythropoietin muteins comprising native erythropoietin with one or more modifications which improves the biological activity.

All references mentioned herein are incorporated by reference into this 20 disclosure.

Having now fully described the invention by way of illustration and example for purposes of clarity and understanding, it will be apparent to those of ordinary skill in the art that certain changes and modifications may be practiced within the scope of the invention, as limited only by the following 25 claims.

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SEQUENCE LISTING

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(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
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(A) APPLICATION NUMBER: (to be assigned)  
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(A) APPLICATION NUMBER: 08/049,802  
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(C) CLASSIFICATION:

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 13 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATWGAATGAA SGC

13

(2) INFORMATION FOR SEQ ID NO:2:

- 72 -

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 10 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

AWGGTNSGGG

10

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 24 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TGCTTCTGCT ATCTTGCTG CTGC

24

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 15 amino acids
  - (B) TYPE: amino acid
  - (D) TOPOLOGY: both

(ix) FEATURE:

- (A) NAME/KEY: Peptide
- (B) LOCATION: 12..13
- (D) OTHER INFORMATION: /label= Peptide  
*/note= "The amino acid at position 12 may also be isoleucine. The amino acid at position 13 may also be aspartic acid."*

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Asp Thr Lys Val Asn Phe Tyr Ala Trp Lys Arg Met Glu Val Gly  
 1               5                   10                   15

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 25 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TGAAGTTTGG CCGAGAAAGTG GATGC

25

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 23 base pairs
  - (B) TYPE: nucleic acid

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- (C) STRANDEDNESS: single
- (D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

AAGAKGTACC TCTCCAGRAC TCG

23

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 22 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CTGCTCCACT CCGAACAMTC AC

22

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 20 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CTGGAGTGTC CATGGGACAG

20

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 20 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

AGGCAGCGGAG ATGGGGGTGC

20

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 286 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TGGTAGCTGG GGGTGGGTG TGCACACGGC AGCAGGATTG AATGAAGGCC AGGGAGGCAG	60
CACCTGAGTG CTTGCATGGT TGGGGACAGG AAGGACCGAGC TGGGGCAGAG ACGTGGGATG	120
AAGGAAGCTG TCCTTCCACA GGCCACCCCTT CTCCCTCCCC GCCTGACTCT CAGCCTGGCT	180
ATCTGTTCTA GAATGTCCTG CCTGGCTGTG GCTTCTCCTG TCCCTGCTGT CGCTCCCTCT	240

- 74 -

GGGCCTCCCA GTCCTGGCG CCCCCACACG CCTCATCTGT GACAGC 286

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 290 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CTCGTAGTTG TTGGGTGGCG TGTGACGCG GTCAGTGTAT TGAATGAAGG CAAAGGAGGC	60
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ATGGGTGAAC CTGTCCGCT AAACCAACCC TTCTCTGCA ATGCCAGCC TCACATTCA	180
CCTGGCTCTC TTTCCTAGAA TGTCTGCCC GGCTGCTTCT GCTATCTTG CTGCTGCTTC	240
CTCTGGGCT CCCAGTCTG GGCGCCCCCC CACGCCAT CTGTGACAGC	290

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 286 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GTAGCTCGGG GGGTGGGTG TGCACGCCGC CGCGGGATTG AATGAAGGCA AAGGAGGCAG	60
AGCCTGAGCG CTCGCAAGGT TCGGGTCGGG AGGACTCGCG GGGCCAGAGC AGCGGGATGG	120
GTGAACGTGC AGGTCAAAC CACCCCTCT CCTGCAAGTC CCCGGCTCAC ACTCAGCCCA	180
GCTCTCTCTC CTAGAATGTC TTCTGCTGCT TCTGCTGCCC TCCCTGCTGC TGCTTCTCT	240
GGGCCTCCCA GTCCTGGCG CCCCCACACA CCTCATCTGT GACAGC	286

(2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 288 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CCGTAGCTGT TGGGGTGGGG TGCGCACGAA GCCGCGGGAT TGAATGAACG CGGAGGCAGA	60
GCTGAGCGCT CCCAAGGTCC GGGTGGGGAG GGCTCACTGG GGGCTGCGCA GCGGGATGGG	120
TGAACCTGCC GGTCCAAGC AGCCCTCTC CTGCGAGAAC GCACCTCATC CGCAGCCCCGT	180
CTCTCTTTCC TAGACTGTAC TCCGCTGCTG CTGCTGCTGC TGTCTCTCT GCTGTTCTC	240

- 75 -

CTGGGCCTCC CAGTCTTGGG CGCCCCCCC CGCCTCATCT GTGACAGC 288

(2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 280 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

TGGGGGTGGG GTGTGCAGGC GGCAGCAGGA TTGAATGAAG GCAGAGGAGA CAGACCCCTGA	60
GGCCTGGGAA GGTTGGGGC AGGAGCCACT AGCTGGGGC AGAGGAGGGG GATGCGTGAA	120
CCTGCCCTC CAAACCAACC CTCCCCCTCC CCTCCCTGCC TCACACTCAG CCCGGATCTC	180
CTTCCAGAA TGTCCTGCC TGCTGCTCT GCTGTCCTG CTACTGCCTC CTCTGGGCCT	240
CCCAGCCCTG GGCGCCCTC CACGCCTCAT CTGTGACACC	280

(2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 274 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CAGGTCAGTT TGATTGGGTG GATGTGTGCC GCGAACGAGC GGCGGGATTG AATGAAGGCA	60
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GGAGCGGGAT GTGTGAACCT GCCCCTCCAA ACCCACACAG TCAGCCTGGC ACTCTTTCC	180
AGAATGTCTT GCCCTGTTC TTTGCTGTC TTTGCTGCTG CTTCCTCTGG ACCTCCCAGT	240
CCTGGGCGCC CCCCTCGCC TCATTTGTGA CAGC	274

(2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 269 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TGGTAGTTG GCGGGTGGTG TGTGCTCACC GCGGCGCGG CGGGGATTGA ATGAAGGCCA	60
AGGAGGCAGA ACCCGAGCGC TGGGAAGGTT CGGGGTGGGA GCGACAAGCT GGGGGCAGAG	120
GAACGGGATG TGTGAACCTG CTCCCTCCAAA CACACTCAGC CTGGCACTCT TTTCTAGAAT	180
GTCTGCCCCCT GCTGCTTCTG CTATCTTGC TGCTGCTTCC CCTGGGCCTC CCAGTCCTGG	240
GCGCCCCCCC TCGCCTCATC TGTGACAGC	269

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(2) INFORMATION FOR SEQ ID NO:17:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 268 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GGTAGTTTGG CGGGTGGGGT GTGCTCACCG CGGGGGCGGC GGGGATTGAA TGAAGGCCAA	60
GGAGGCAGAA CCCGAGCGCT GGGAAAGGTTTC GGGGTGGGAG CGACAAGCTG GGGGCAGAGG	120
AACGGGATGT GTGAACCTGC TGCTCAAAC ACACTCAGCC TGGCACTCTT TTCTAGAATG	180
TCCTGCCCTG CTGCTTCTGC TATCTTGCT GCTGCTTCCC CTGGGCCTCC CAGTCCTGGG	240
CGCCCCCCCCCT CGCCTCATCT GTGACAGC	268

(2) INFORMATION FOR SEQ ID NO:18:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 283 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CGGTCGCTTG GGGGGTGGGG TGTGCAGCGC GGAGGGATTG AATGAAGGCC ACTCAGGCAG	60
AGCCTAACGCA ATTGCAAGGT CGGGGTCAAGC AGAGACTATA AGGGCAGAGG GGTCTCGCTG	120
AGCCAACCGG CCCCTGCAGT CCCACGGCCC CCTCCCCCTCT CGGCCTCACA CTCAGCCTGC	180
CTTTCTTCCA GAACGTCCCA CCCTGCTGCT TTTACTCTCC TTGCTACTGA TTCCTCTGGG	240
CCTCCCAAGTC CTCTGTGCTC CCCCCACGCCT CATCTGCGAC AGT	283

(2) INFORMATION FOR SEQ ID NO:19:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 283 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

ATGGTGGCTT GGGGGGTGGT GTGTGCAGCG CGGGGAGATA GAATGAAGGC CACTCAGGCA	60
GAGCCTAACGC AGTTGCAAGG TCGGGGTCAAG CAGAGACTGG AAGGGCAGAG GAGCCTCGCT	120
GTGCAAACCG GTCTCTGGTC GTCCCAACGGC CCTCCCCCTCC CAGCCTCACA CCCAGCCTGC	180
CTTTCTTCTA GAACGTCCCA CCCTGCTGCT TTTGCTGTCT TTGCTCCTGC TTCCCCCTGGG	240
CCTCCCAAGTC CTCTGTGCTC CCCCCACGCCT CATCTGCGAC AGC	283

(2) INFORMATION FOR SEQ ID NO:20:

- (i) SEQUENCE CHARACTERISTICS:

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- (A) LENGTH: 112 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: both

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GGGTGGTGGC GATGAATGAA GCAGCAGCTA GGTGGGGGGC GGTGAAGTAC AGCCGTCTCA	60
GAGTGTCTTC TCTCTGCCCT GGCCCTCCAG CTGGCCCCCC CTCATTGGAC AG	112

## (2) INFORMATION FOR SEQ ID NO:21:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 725 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: both

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

AGGCAGCGGAG ATGGGGGTGC ACGAATGTCC TGCCCTGGCTG TGGCTTCTCC TGTCCCTGCT	60
GTGGCTCCCT CTGGGCCCTCC CAGTCCTGGG CGCCCCCACCA CGCCTCATCT GTGACAGCCG	120
AGTCCTGGAG AGGTACCTCT TGGAGGCCAA GGAGGCCAG AATATCACGA CGGGCTGTGC	180
TGAACACTGC AGCTTGAATG AGAATATCAC TGTCCCAGAC ACCAAAGTTA ATTTCTATGC	240
CTGGAAGAGG ATGGAGGTGCG GCCAGCAGGC CGTAGAACGTC TGGCAGGGCC TGGCCCTGCT	300
GTGGAAAGCT GTCCCTGCCGG GCCAGGCCCT GTTGGTCAAC TCTTCCCAGC CGTGGGAGCC	360
CCTGCAGCTG CTTGTGGATA AAGCCGTCAAG TGGCCTTCGC AGCCTCACCA CTCTGCTTCG	420
GGCTCTGGGA GCCCAGAAAG AAGCCATCTC CCCTCCAGAT GCGGCCCTCAG CTGCTCCACT	480
CCGAACAATC ACTGCTGACA CTTTCCGCAA ACTCTTCCGA GTCTACTCCA ATTTCTCCG	540
GGGAAAGCTG AAGCTGTACA CAGGGGAGGC CTGCAGGACA GGGGACAGAT GACCAGGTGT	600
GTCCACCTGG GCATATCCAC CACCTCCCTC ACCAACATTG CTTGTGCCAC ACCCTCCCCC	660
GGCCACTCCT GAACCCCGTC GAGGGCTCT CAGCTCAGCG CCAGCCTGTC CCATGGACAC	720
TCCAG	725

## (2) INFORMATION FOR SEQ ID NO:22:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 681 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: both

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

ACGAATGTCC TGCCCTGGCTG TGGCTTCTCC TGTCTCTCGT GTGGCTCCCT CTGGGCCCTCC	60
CAGTCCCGGG CGCCCCCACCA CGCCTCGTCT GTGACAGCCG AGTCCTGGAG AGGTACCTCT	120
TGGAGGCCAA GGAGGCCAG AATGTACAGA TGGCTGTTC CGAAAGCTGC AGCTTGAATG	180

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AGAATATCAC CGTCCCAGAC ACCAAAGTTA ACTTCTATGC CTGGAAGAGG ATAGAGGTG	240
GGCAGCAGGC TGTAGAAGTC TGGCAGGGCC TGGCCCTGCT CTCAGAAGCT GTCTGCAGGG	300
GCCAGGCCGT GTGGCCAAC TCTTCCCAGC CTTTCGAGCC CCTGCAGCTG CACATGGATA	360
AAGCCATCAG TGGCCTTCGC AGCATCACCA CTCTGCTTCG GGCCTGGGA GCCCAGGAAG	420
CCATCTCCCT CCCAGATGCG GCCTCGGCTG CTCCACTCCG AACCCTCACT GCTGACACTT	480
TCTGCAAACCT CTTCCGAGTC TACTCCAATT TCCTCCGGGG AAAGCTGAAG CTGTACACGG	540
GGGAGGCCTG CAGGAGAGGG GACAGATGAC CAGGTGCGTC CAGCTGGCA CATCCACCAA	600
CTCCCTCACC AACACTGCCT GTGCCACACC CTCCCTCACC ACTCCCCAAC CCCATCGAGG	660
GGCTCTCAGC TAAGCGCCAG C	681

(2) INFORMATION FOR SEQ ID NO:23:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 679 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

CCGAACGTCC CACCTGCTG CTTTACTCT CCTTGCTACT GATTCTCTG GGCCTCCCAG	60
TCCTCTGTGC TCCCCCACGC CTCATCTGG ACAGTCGAGT TCTGGAGAGG TACATCTTAG	120
AGGCCAAGGA GGCAGAAAAT GTCACGATGG GTTGTGCAGA AGGTCCCAGA CTGAGTGAAA	180
ATATTACAGT CCCAGATACC AAAGTCAACT TCTATGCTTG GAAAAGAATG GAGGTGGAAG	240
AAACAGGCCAT AGAAGTTTGG CAAGGCCTGT CCCTGCTCTC AGAACCCATC CTGCAGGCC	300
AGGCCCTGCT AGCCAATTCC TCCCAGCCAC CAGAGACCTC TCAGCTTCAT ATAGACAAAG	360
CCATCAGTGG TCTACGTAGC CTCACITTCAC TGCTCCGGGT ACTGGGAGCT CAGAAGGAAT	420
TGATGTGCC TCCAGATACC ACCCCACCTG CTCCACTCCG AACACTCACA GTGGATACTT	480
TCTGCAAGCT CTTCCGGGTC TACGCCAATC TCCTCCGGGG GAAACTGAAG CTGTACACGG	540
GAGAGGTCTG CAGGAGAGGG GACAGGTGAC ATGCTGCTGC CACCGTGGTG GACCGACGAA	600
CTTGCTCCCC GTCACTGTGT CATGCCAACC CTCCACCACT CCCAACCCCTC ATCAAACGGG	660
TCATTACCTT CTTACCACT	679

(2) INFORMATION FOR SEQ ID NO:24:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 678 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

CCGAACGTCC CACCTGCTG CTTTACTAT CCTTGCTACT GATTCTCTG GGCCTCCCAG	60
TCCTCTGCGC TCCCCCACGC CTCATTTGGC ACAGTCGGGT TCTGGAGAGG TACATCTGG	120

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AGGCCAAGGA GGCAGAAAAT GTCACAATGG GCTGTGCAGA AGGTCCCAGA CTGACTGAGA	180
ATATTACCGT CCCAGATAACC AAAGTCAACT TCTACGCTTG GAAAAGAATG AAGGTGGAAG	240
AACAGGCTGT AGAACGTTGG CAAGGCCGT CTCTGCTCTC AGAACGCCATC CTGCAGGCC	300
AGGCTCTGCA GGCCAATTCC TCCCAGCCAC CAGAGAGTCT TCAGCTTCAT ATAGACAAAG	360
CCATCAGTGG GCTACGTAGC CTCACCTCAC TGCTTGGGT GCTGGGAGCT CAGAAGGAAT	420
TGATGTCGCC TCCAGACGCC ACCCAAGCCG CTCCACTCCG AACACTCACA GCGGATACTT	480
TCTGCAAGCT CTTCCGGGTC TACTCCAATC TCCTCCGGGG GAAACTGAAG CTGTACACGG	540
GGGAGGCCCTG CAGGAGAGGG GACAGGTGAC CTGCCACTGC CGTGTACCCG CCAACTCGCT	600
CACCGTCACT GTGTCACGCC AACCCCTCAC CACTCCAAC CCTCATCAAA CGGGGTTGTT	660
TGTTACCTTC TTACCGGC	678

(2) INFORMATION FOR SEQ ID NO:25:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 687 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

GCGACTGTAC TCCGCTGCTG CTGCTGCTGC TGTCTCTTCT GCTGTTTCT CTGGGCCTCC	60
CAGTCTTGGG CGCCCCCCCCA CGCCTCATCT GTGACAGCCG AGTCTGGAG AGGTACATCC	120
TGGAGGCCAG GGAGGCCGAA AATGCCACGA TGGCTGTGC AGAAGGCTGC AGCTTCAGTG	180
AGAATATCAC TGTCCAGAC ACCAAGGTTA ACTTCTATGC CTGGAAGAGG ATGGAGGTCC	240
AGCAGCAGGC TCTGGAAGTC TGGCAGGGCC TGGCTCTGCT CTCAGAAGCT ATCTTTCGGG	300
GCCAGGCCCT ACCGGCCAAC GCATCCCAGC CATGCCAGGC CCTGCGGTTG CACGTGGACA	360
AAGCTGTCAG CGGCCCTCCCG AGTCTCACCT CCCTGCTTCTG GGGCTGGGA GCCCAGAAAG	420
AAGCCATCCC CCTTCCAGAT GCAACCCCT CGCGAGCCCC ACTCCGAATA TTCACTGTTG	480
ATGCTTGTGTC CAAGCTCTTC CGAATCTACT CCAATTCTCT GAGGGGAAAG CTGACGCTGT	540
ACACAGGGGA GGCCTGCAGG AGAGGGGACA GGTGACCCAG TGCTATCACC CCGGGCACGT	600
CCATCACCTC GCTCACCATC ACTGCCTACG CCATGCCTTC CACGCCGCCA CTCCCAACCC	660
CTGTCGACGA CGGGCCACCA GCTCACT	687

(2) INFORMATION FOR SEQ ID NO:26:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 688 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

GAATGTCCTG CCCGGCTGCT TCTGCTATCT TTGCTGCTGC TTCCCTCTGGG CCTCCCAAGTC	60
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CTGGGCGCCC	CCCCACGCCT	CATCTGTGAC	AGCCGAGTCC	TGGAGAGGTA	CATCTGGAG	120
GCCAAGGAGG	GCGAAAATGC	CACGATGGC	TGTGCCAAA	GCTGCAGCTT	CAGTGAGAAT	180
ATCACTGTCC	CAGACACCAA	GGTTAACCTTC	TATGCCCTGGA	AGAGGATGGA	GGTCCAGCAG	240
CAGGCCATGG	AGGTCTGGCA	GGGTCTGGCC	CTGCTCTCAG	AAGCCATCCT	GCAGGGCCAG	300
GCCCTGTTGG	CCAACCTCTC	CCAGCCATCT	GAGGCCCTGC	AGCTGCATGT	GGACAAAGCT	360
GTCAGCGGCC	TGGCAGCCT	CACCTCCCTG	CTTCGGGCC	TGGGAGCCCA	GAAGGAAGCC	420
ATCCCCCTTC	CAGACGCATC	CCCCTCCCT	GCCACCCAC	TCCGAACATT	TGCTGTTGAT	480
ACTTTGTGCA	AACTTTCCG	CAACTACTCC	AATTCTCTGC	GGGGAAAGCT	GACGCTGTAC	540
ACTGGAGAGG	CCTGCAGGAG	AAGGGACAGG	TGACTTGGTG	CTCCCACCCG	GGGCATGTCC	600
ACCACTTCGC	TCACCACAC	TGCCCTGTCC	ATGCCCTCTG	CACCTCCACT	CCCAACCCCC	660
GCTGAGGGGC	CATCAGCTCA	GCGCCAGC				688

(2) INFORMATION FOR SEQ ID NO:27:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 681 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

GAATGTCTG	CCCTGCTGCT	TCTGCTATCT	TTGCTGCTGC	TTCCCCCTGGG	CCTCCCGAGTC	60
CTGGGCGCCC	CCCCCTCGCCT	CATCTGTGAC	AGCCGAGTCC	TGGAGAGGTA	CATTCTGGAG	120
GCCAGGGAGG	CCGAAAATGT	CACGATGGC	TGTGCTGAAG	GCTGCAGCTT	CAGTGAGAAT	180
ATCACTGTCC	CAGACACCAA	GGTCAACTTC	TATACTGGA	AGAGGATGCA	CGTCGGGCAG	240
CAGGCTGTGG	AAGTCTGGCA	GGGCCTCGCC	CTGCTCTCAG	AAGCCATCCT	GGGGGGCCAG	300
GCCCTGCTGG	CCAACCTCTC	CCAGCCATCT	GAGACCCCTGC	AGCTGCATGT	GGATAAAAGCC	360
GTCAGCAGCC	TGGCAGCCT	CACCTCCCTG	CTTCGGGCAC	TGGGAGCCCA	GAAGGAAGCC	420
ACCTCCCTTC	CAGAGGCAAC	CTCTGCTGCT	CCACTCCGAA	CATTCACTGT	CGATACTTTG	480
TGCAAACTTT	TCCGAATCTA	CTCCAACCTTC	CTGCGGGGAA	AGCTGACGCT	GTACACAGGG	540
GAGGCCTGCC	GAAGAGGAGA	CAGGTGACCA	GGTGCTCCTA	CCCCGGGCAT	GTCCACCACCC	600
TCACATTACCA	CCAATGCCTG	TGCCACGCC	TCTGCACCAAC	CACTCCTGAC	CCCTGTCGGG	660
GGTGATCAGC	TCAGCACCAAGC	C				681

(2) INFORMATION FOR SEQ ID NO:28:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 30 amino acids
  - (B) TYPE: amino acid
  - (D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

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Met Gly Val His Glu Cys Pro Ala Trp Leu Trp Leu Leu Ser Leu  
1 5 10 15  
Leu Ser Leu Pro Leu Gly Leu Pro Val Leu Gly Ala Pro Pro  
20 25 30

(2) INFORMATION FOR SEQ ID NO:29:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 30 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

Met Gly Val His Glu Cys Pro Ala Trp Leu Trp Leu Leu Ser Leu  
1 5 10 15  
Val Ser Leu Pro Leu Gly Leu Pro Val Pro Gly Ala Pro Pro  
20 25 30

(2) INFORMATION FOR SEQ ID NO:30:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 29 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

Met Gly Val Pro Glu Arg Pro Thr Leu Leu Leu Leu Ser Leu Leu  
1 5 10 15  
Leu Ile Pro Leu Gly Leu Pro Val Leu Cys Ala Pro Pro  
20 25

(2) INFORMATION FOR SEQ ID NO:31:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 30 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

Met Gly Val Arg Asp Cys Thr Pro Leu Leu Leu Leu Leu Ser Leu  
1 5 10 15  
Leu Leu Phe Pro Leu Gly Leu Pro Val Leu Gly Ala Pro Pro  
20 25 30

(2) INFORMATION FOR SEQ ID NO:32:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 25 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

Glu Cys Pro Ala Arg Leu Leu Leu Ser Leu Leu Leu Pro Leu

- 82 -

1                   5                   10                   15

Gly Leu Pro Val Leu Gly Ala Pro Pro  
20   25

(2) INFORMATION FOR SEQ ID NO:33:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 25 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

Glu Cys Pro Ala Leu Leu Leu Leu Ser Leu Leu Pro Pro Leu  
1                   5   10   15

Gly Leu Pro Ala Leu Gly Ala Pro Pro  
20   25

(2) INFORMATION FOR SEQ ID NO:34:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 25 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

Glu Cys Pro Ala Leu Leu Leu Leu Ser Leu Leu Leu Pro Leu  
1                   5   10   15

Gly Leu Pro Val Leu Gly Ala Pro Pro  
20   25

(2) INFORMATION FOR SEQ ID NO:35:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 25 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

Glu Cys Pro Ala Leu Phe Leu Leu Leu Ser Leu Leu Leu Pro Leu  
1                   5   10   15

Asp Leu Pro Val Leu Gly Ala Pro Pro  
20   25

(2) INFORMATION FOR SEQ ID NO:36:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 25 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

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Glu Cys Leu Leu Leu Leu Leu Pro Ser Leu Leu Leu Leu Pro Leu  
 1 5 10 15  
 Gly Leu Pro Val Leu Gly Ala Pro Pro  
 20 25

(2) INFORMATION FOR SEQ ID NO:37:

- (i) SEQUENCE CHARACTERISTICS:

  - (A) LENGTH: 166 amino acids
  - (B) TYPE: amino acid
  - (D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

Ala Pro Pro Arg Leu Ile Cys Asp Ser Arg Val Leu Glu Arg Tyr Leu  
 1 5 10 15

Leu Glu Ala Lys Glu Ala Glu Asn Ile Thr Thr Gly Cys Ala Glu His  
 20 25 30

Cys Ser Leu Asn Glu Asn Ile Thr Val Pro Asp Thr Lys Val Asn Phe  
 35 40 45

Tyr Ala Trp Lys Arg Met Glu Val Gly Gln Gln Ala Val Glu Val Trp  
 50 55 60

Gln Gly Leu Ala Leu Leu Ser Glu Ala Val Leu Arg Gly Gln Ala Leu  
 65 70 75 80

Leu Val Asn Ser Ser Gln Pro Trp Glu Pro Leu Gln Leu His Val Asp  
 85 90 95

Lys Ala Val Ser Gly Leu Arg Ser Leu Thr Thr Leu Leu Arg Ala Leu  
 100 105 110

Gly Ala Gln Lys Glu Ala Ile Ser Pro Pro Asp Ala Ala Ser Ala Ala  
 115 120 125

Pro Leu Arg Thr Ile Thr Ala Asp Thr Phe Arg Lys Leu Phe Arg Val  
 130 135 140

Tyr Ser Asn Phe Leu Arg Gly Lys Leu Lys Leu Tyr Thr Gly Glu Ala  
 145 150 155 160

Cys Arg Thr Gly Asp Arg  
 165

(2) INFORMATION FOR SEQ ID NO:38:

- (i) SEQUENCE CHARACTERISTICS:

  - (A) LENGTH: 165 amino acids
  - (B) TYPE: amino acid
  - (D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

Ala	Pro	Pro	Arg	Leu	Ile	Cys	Asp	Ser	Arg	Val	Leu	Glu	Arg	Tyr	Leu
1				5					10					15	
Leu	Glu	Ala	Lys	Glu	Ala	Glu	Asn	Val	Thr	Met	Gly	Cys	Ser	Glu	Ser
			20					25					30		
Cys	Ser	Leu	Asn	Glu	Asn	Ile	Thr	Val	Pro	Asp	Thr	Lys	Val	Asn	Phe
			35				40					45			

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Tyr Ala Trp Lys Arg Met Glu Val Gly Gln Gln Ala Val Glu Val Trp  
 50                       55                       60

Gln Gly Leu Ala Leu Leu Ser Glu Ala Val Leu Arg Gly Gln Ala Val  
 65                       70                       75                       80

Leu Ala Asn Ser Ser Gln Pro Phe Glu Pro Leu Gln Leu His Met Asp  
 85                       90                       95

Lys Ala Ile Ser Gly Leu Arg Ser Ile Thr Thr Leu Leu Arg Ala Leu  
 100                      105                       110

Gly Ala Gln Glu Ala Ile Ser Leu Pro Asp Ala Ala Ser Ala Ala Pro  
 115                      120                       125

Leu Arg Thr Ile Thr Ala Asp Thr Phe Cys Lys Leu Phe Arg Val Tyr  
 130                      135                       140

Ser Asn Phe Leu Arg Gly Lys Leu Lys Leu Tyr Thr Gly Glu Ala Cys  
 145                      150                       155                       160

Arg Arg Gly Asp Arg  
 165

(2) INFORMATION FOR SEQ ID NO:39:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 165 amino acids
  - (B) TYPE: amino acid
  - (D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

Ala Pro Pro Arg Leu Val Cys Asp Ser Arg Val Leu Glu Arg Tyr Leu  
 1                       5                           10                       15

Leu Glu Ala Lys Glu Ala Glu Asn Val Thr Met Gly Cys Ser Glu Ser  
 20                      25                           30

Cys Ser Leu Asn Glu Asn Ile Thr Val Pro Asp Thr Lys Val Asn Phe  
 35                      40                           45

Tyr Ala Trp Lys Arg Ile Glu Val Gly Gln Gln Ala Val Glu Val Trp  
 50                      55                           60

Gln Gly Leu Ala Leu Leu Ser Glu Ala Val Leu Arg Gly Gln Ala Val  
 65                      70                           75                       80

Leu Ala Asn Ser Ser Gln Pro Phe Glu Pro Leu Gln Leu His Met Asp  
 85                      90                           95

Lys Ala Ile Ser Gly Leu Arg Ser Ile Thr Thr Leu Leu Arg Ala Leu  
 100                     105                       110

Gly Ala Gln Glu Ala Ile Ser Leu Pro Asp Ala Ala Ser Ala Ala Pro  
 115                      120                       125

Leu Arg Thr Ile Thr Ala Asp Thr Phe Cys Lys Leu Phe Arg Val Tyr  
 130                      135                       140

Ser Asn Phe Leu Arg Gly Lys Leu Lys Leu Tyr Thr Gly Glu Ala Cys  
 145                      150                       155                       160

Arg Arg Gly Asp Arg  
 165

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(2) INFORMATION FOR SEQ ID NO:40:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 166 amino acids
  - (B) TYPE: amino acid
  - (D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

Ala	Pro	Pro	Arg	Leu	Ile	Cys	Asp	Ser	Arg	Val	Leu	Glu	Arg	Tyr	Ile
1															15
Leu	Glu	Ala	Lys	Glu	Ala	Glu	Asn	Val	Thr	Met	Gly	Cys	Ala	Glu	Gly
															30
Pro	Arg	Leu	Ser	Glu	Asn	Ile	Thr	Val	Pro	Asp	Thr	Lys	Val	Asn	Phe
															45
Tyr	Ala	Trp	Lys	Arg	Met	Glu	Val	Glu	Gln	Ala	Ile	Glu	Val	Trp	
															50
Gln	Gly	Leu	Ser	Leu	Leu	Ser	Glu	Ala	Ile	Leu	Gln	Ala	Gln	Ala	Leu
65															80
Leu	Ala	Asn	Ser	Ser	Gln	Pro	Pro	Glu	Thr	Leu	Gln	Leu	His	Ile	Asp
															95
Lys	Ala	Ile	Ser	Gly	Leu	Arg	Ser	Leu	Thr	Ser	Leu	Leu	Arg	Val	Leu
															110
Gly	Ala	Gln	Lys	Glu	Leu	Met	Ser	Pro	Pro	Asp	Thr	Thr	Pro	Pro	Ala
															125
Pro	Leu	Arg	Thr	Leu	Thr	Val	Asp	Thr	Phe	Cys	Lys	Leu	Phe	Arg	Val
															130
Tyr	Ala	Asn	Phe	Leu	Arg	Gly	Lys	Leu	Lys	Leu	Tyr	Thr	Gly	Glu	Val
															145
Cys	Arg	Arg	Gly	Asp	Arg										
															165

(2) INFORMATION FOR SEQ ID NO:41:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 166 amino acids
  - (B) TYPE: amino acid
  - (D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

Ala	Pro	Pro	Arg	Leu	Ile	Cys	Asp	Ser	Arg	Val	Leu	Glu	Arg	Tyr	Ile	
1															15	
Leu	Glu	Ala	Lys	Glu	Ala	Glu	Asn	Val	Thr	Met	Gly	Cys	Ala	Glu	Gly	
															30	
Pro	Arg	Leu	Ser	Glu	Asn	Ile	Thr	Val	Pro	Asp	Thr	Lys	Val	Asn	Phe	
															45	
Tyr	Ala	Trp	Lys	Arg	Met	Lys	Val	Glu	Glu	Gln	Ala	Val	Glu	Val	Trp	
															50	
Gln	Gly	Leu	Ser	Leu	Leu	Ser	Glu	Ala	Ile	Leu	Gln	Ala	Gln	Ala	Leu	

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65	70	75	80
Gln			
Ala	Asn	Ser	Ser
Gln	Pro	Pro	Glu
			Leu
		Gln	Leu
			His
			Ile
			Asp
85		90	95
Lys	Ala	Ile	Ser
Gly	Leu	Arg	Ser
	Leu	Thr	Ser
		Leu	Leu
		Arg	Val
100		105	110
Gly	Ala	Gln	Lys
Glu	Leu	Met	Ser
	Pro	Pro	Asp
115		120	125
Pro	Leu	Arg	Thr
Thr	Leu	Ala	Asp
		Thr	Phe
		Cys	Lys
			Leu
			Phe
			Arg
130		135	140
Tyr	Ser	Asn	Phe
			Leu
			Arg
		Gly	Lys
		Leu	Lys
			Leu
145		150	155
			160
Cys	Arg	Arg	Gly
			Asp
			Arg
165			

(2) INFORMATION FOR SEQ ID NO:42:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 167 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

Ala	Pro	Pro	Arg	Leu	Ile	Cys	Asp	Ser	Arg	Val	Glu	Arg	Tyr	Ile	
1				5					10			15			
Leu	Glu	Ala	Arg	Glu	Ala	Glu	Asn	Ala	Thr	Met	Gly	Cys	Ala	Glu	Gly
	20				25				30						
Cys	Ser	Phe	Ser	Glu	Asn	Ile	Thr	Val	Pro	Asp	Thr	Lys	Val	Asn	Phe
	35				40			45							
Tyr	Ala	Trp	Lys	Arg	Met	Glu	Val	Gln	Gln	Ala	Leu	Glu	Val	Trp	
	50				55			60							
Gln	Gly	Leu	Ala	Leu	Leu	Ser	Glu	Ala	Ile	Phe	Arg	Gly	Gln	Ala	Leu
65					70			75			80				
Pro	Ala	Asn	Ala	Ser	Gln	Pro	Cys	Glu	Ala	Leu	Arg	Leu	His	Val	Asp
	85				90			95							
Lys	Ala	Val	Ser	Gly	Leu	Arg	Ser	Leu	Thr	Ser	Leu	Leu	Arg	Ala	Leu
	100				105			110							
Gly	Ala	Gln	Lys	Glu	Ala	Ile	Pro	Leu	Pro	Asp	Ala	Thr	Pro	Ser	Ala
	115				120			125							
Ala	Pro	Leu	Arg	Ile	Phe	Thr	Val	Asp	Ala	Leu	Ser	Lys	Leu	Phe	Arg
	130				135			140							
Ile	Tyr	Ser	Asn	Phe	Leu	Arg	Gly	Lys	Leu	Thr	Leu	Tyr	Thr	Gly	Glu
145				150				155				160			
Ala	Cys	Arg	Arg	Gly	Asp	Arg									
	165														

(2) INFORMATION FOR SEQ ID NO:43:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 168 amino acids
- (B) TYPE: amino acid

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(D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

Ala	Pro	Pro	Arg	Leu	Ile	Cys	Asp	Ser	Arg	Val	Leu	Glu	Arg	Tyr	Ile
1				5					10				15		
Leu	Glu	Ala	Lys	Glu	Gly	Glu	Asn	Ala	Thr	Met	Gly	Cys	Ala	Glu	Ser
	20				25				30						
Cys	Ser	Phe	Ser	Glu	Asn	Ile	Thr	Val	Pro	Asp	Thr	Lys	Val	Asn	Phe
	35				40			45							
Tyr	Ala	Trp	Lys	Arg	Met	Glu	Val	Gln	Gln	Gln	Ala	Met	Glu	Val	Trp
	50				55			60							
Gln	Gly	Leu	Ala	Leu	Leu	Ser	Glu	Ala	Ile	Leu	Gln	Gly	Gln	Ala	Leu
65				70					75				80		
Leu	Ala	Asn	Ser	Ser	Gln	Pro	Ser	Glu	Ala	Leu	Gln	Leu	His	Val	Asp
		85						90					95		
Lys	Ala	Val	Ser	Gly	Leu	Arg	Ser	Leu	Thr	Ser	Leu	Leu	Arg	Ala	Leu
		100				105				110					
Gly	Ala	Gln	Lys	Glu	Ala	Ile	Pro	Leu	Pro	Asp	Ala	Ser	Pro	Ser	Ser
		115				120				125					
Ala	Thr	Pro	Leu	Arg	Thr	Phe	Ala	Val	Asp	Thr	Leu	Cys	Lys	Leu	Phe
		130				135				140					
Arg	Asn	Tyr	Ser	Asn	Phe	Leu	Arg	Gly	Lys	Leu	Thr	Leu	Tyr	Thr	Gly
145				150				155					160		
Glu	Ala	Cys	Arg	Arg	Arg	Asp	Arg								
			165												

(2) INFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 166 amino acids
- (B) TYPE: amino acid
- (C) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

Ala	Pro	Pro	Arg	Leu	Ile	Cys	Asp	Ser	Arg	Val	Leu	Glu	Arg	Tyr	Ile
1				5					10				15		
Leu	Glu	Ala	Arg	Glu	Ala	Glu	Asn	Val	Thr	Met	Gly	Cys	Ala	Glu	Gly
	20				25				30						
Cys	Ser	Phe	Ser	Glu	Asn	Ile	Thr	Val	Pro	Asp	Thr	Lys	Val	Asn	Phe
	35				40			45							
Tyr	Thr	Trp	Lys	Arg	Met	Asp	Val	Gly	Gln	Ala	Val	Glu	Val	Trp	
	50				55			60							
Gln	Gly	Leu	Ala	Leu	Leu	Ser	Glu	Ala	Ile	Leu	Arg	Gly	Gln	Ala	Leu
65				70					75				80		
Leu	Ala	Asn	Ser	Ser	Gln	Pro	Ser	Glu	Thr	Leu	Gln	Leu	His	Val	Asp
		85						90					95		

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Lys Ala Val Ser Ser Leu Arg Ser Leu Thr Ser Leu Leu Arg Ala Leu  
 100 105 110

Gly Ala Gln Lys Glu Ala Thr Ser Leu Pro Glu Ala Thr Ser Ala Ala  
 115 120 125

Pro Leu Arg Thr Phe Thr Val Asp Thr Leu Cys Lys Leu Phe Arg Ile  
 130 135 140

Tyr Ser Asn Phe Leu Arg Gly Lys Leu Thr Leu Tyr Thr Gly Glu Ala  
 145 150 155 160

Cys Arg Arg Gly Asp Arg  
 165

(2) INFORMATION FOR SEQ ID NO:45:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 166 amino acids
  - (B) TYPE: amino acid
  - (D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

Ala Pro Pro Arg Leu Ile Cys Asp Ser Arg Val Leu Glu Arg Tyr Leu  
 1 5 10 15

Leu Glu Ala Lys Glu Ala Glu Asn Ile Thr Thr Gly Cys Ala Glu His  
 20 25 30

Cys Ser Leu Asn Glu Asn Ile Thr Val Pro Asp Thr Lys Val Asn Phe  
 35 40 45

Tyr Ala Trp Lys Arg Met Glu Gly Val Gln Gln Ala Val Glu Val Trp  
 50 55 60

Gln Gly Leu Ala Leu Leu Ser Glu Ala Val Leu Arg Gly Gln Ala Leu  
 65 70 75 80

Leu Val Asn Ser Ser Gln Pro Trp Glu Pro Leu Gln Leu His Val Asp  
 85 90 95

Lys Ala Val Ser Gly Leu Arg Ser Leu Thr Thr Leu Leu Arg Ala Leu  
 100 105 110

Gly Ala Gln Lys Glu Ala Ile Ser Pro Pro Asp Ala Ala Ser Ala Ala  
 115 120 125

Pro Leu Arg Thr Ile Thr Ala Asp Thr Phe Arg Lys Leu Phe Arg Val  
 130 135 140

Tyr Ser Asn Phe Leu Arg Gly Lys Leu Lys Leu Tyr Thr Gly Glu Ala  
 145 150 155 160

Cys Arg Thr Gly Asp Arg  
 165

(2) INFORMATION FOR SEQ ID NO:46:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 14 amino acids
  - (B) TYPE: amino acid
  - (D) TOPOLOGY: both

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

Ser Arg Val Leu Glu Arg Tyr Leu Leu Glu Xaa Xaa Ala Lys  
1 5 10

(2) INFORMATION FOR SEQ ID NO:47:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 18 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

Gln Ala Val Glu Val Trp Gln Gly Leu Ala Leu Leu Ser Glu Ala Val  
1 5 10 15

Leu Arg

(2) INFORMATION FOR SEQ ID NO:48:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 18 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

Pro Leu Gln Leu His Val Asp Lys Ala Val Ser Gly Leu Arg Ser Leu  
1 5 10 15

Thr Thr

(2) INFORMATION FOR SEQ ID NO:49:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 21 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

Thr Ile Thr Ala Asp Thr Phe Arg Lys Leu Phe Arg Val Tyr Ser Asn  
1 5 10 15

Phe Leu Arg Gly Lys  
20

(2) INFORMATION FOR SEQ ID NO:50:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 36 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

- 90 -

Glu Asn Ile Thr Thr Gly Cys Ala Glu His Cys Ser Leu Asn Glu Asn  
1 5 10 15

Ile Thr Val Pro Asp Thr Lys Val Asn Phe Tyr Ala Trp Lys Arg Asn  
20 25 30

Glu Val Gly Gln  
35

(2) INFORMATION FOR SEQ ID NO:51:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 27 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

Leu Thr Thr Leu Leu Arg Ala Leu Gly Ala Gln Lys Glu Ala Ile Ser  
1 5 10 15

Pro Pro Asp Ala Ala Ser Ala Ala Pro Leu Arg  
20 25

(2) INFORMATION FOR SEQ ID NO:52:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 34 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:

Leu Thr Thr Leu Leu Arg Ala Leu Gly Ala Gln Lys Leu Ile Ser Glu  
1 5 10 15

Glu Asp Leu Glu Ala Ile Ser Pro Pro Asp Ala Ala Ser Ala Ala Pro  
20 25 30

Leu Arg

(2) INFORMATION FOR SEQ ID NO:53:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 6 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:53:

Cys Arg Thr Gly Asp Arg  
1 5

(2) INFORMATION FOR SEQ ID NO:54:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 4 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: both

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:54:

Lys Asp Glu Leu  
1

(2) INFORMATION FOR SEQ ID NO:55:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 6 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:55:

His His His His His  
1 5

(2) INFORMATION FOR SEQ ID NO:56:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 16 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:56:

Met Gly His Ser Ser Gly His Ile Glu Gly Arg His Met Ala Pro Pro  
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:57:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 12 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:57:

Val Leu Glu Arg Tyr Leu Leu Glu Ala Lys Glu Ala  
1 5 10

(2) INFORMATION FOR SEQ ID NO:58:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 20 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:58:

Arg Thr Ile Thr Ala Asp Thr Phe Arg Lys Leu Phe Arg Val Tyr Ser  
1 5 10 15

Asn Phe Leu Arg  
20

(2) INFORMATION FOR SEQ ID NO:59:

- (i) SEQUENCE CHARACTERISTICS:

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- (A) LENGTH: 11 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:59:

Gly Lys Leu Lys Leu Tyr Thr Gly Glu Ala Cys  
1               5                   10

**Claims:**

1        1. An erythropoietin mutein comprising native erythropoietin with  
2 one or more of the following modifications:

- 3              (a) replacement of the amino acid at position 20 with a first  
4 substitute amino acid;  
5              (b) replacement of the amino acid at position 49 with a  
6 second substitute amino acid;  
7              (c) replacement of the amino acid at position 73 with a  
8 third substitute amino acid;  
9              (d) replacement of the amino acid at position 140 with a  
10 fourth substitute amino acid;  
11              (e) replacement of the amino acid at position 143 with a  
12 fifth substitute amino acid; and  
13              (f) replacement of the amino acid at position 146 with a  
14 sixth substitute amino acid;  
15              (g) replacement of the amino acid at position 147 with a  
16 seventh substitute amino acid;  
17              (h) replacement of the amino acid at position 154 with a  
18 eighth substitute amino acid;

19        wherein said erythropoietin mutein exhibits enhanced biological activity  
20 compared to said native erythropoietin.

1        2. The erythropoietin mutein of claim 1 comprising native  
2 erythropoietin with one of said modifications.

1        3. The erythropoietin mutein of claim 1 wherein said first, second,  
2 third, fourth, fifth, sixth, seventh or eighth substitute amino acids are selected  
3 from the group consisting of alanine, serine, threonine, and glycine.

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1        4.    The erythropoietin mutein of claim 3 wherein said first  
2    substitute amino acid is serine, and/or said second, third, fourth, fifth, sixth,  
3    seventh and/or eighth substitute amino acids are alanine.

5.    The erythropoietin mutein of claim 2 wherein the amino acid at  
position 20 is replaced with the amino acid alanine.

1        6.    The erythropoietin mutein of claim 2 wherein the amino acid at  
2    position 49 is replaced with the amino acid serine.

3        7.    The erythropoietin mutein of claim 2 wherein the amino acid at  
4    position 73 is replaced with the amino acid glycine.

5        8.    The erythropoietin mutein of claim 2 wherein the amino acid at  
6    position 140 is replaced with the amino acid alanine.

7        9.    The erythropoietin mutein of claim 2 wherein the amino acid at  
8    position 143 is replaced with the amino acid alanine.

9        10.   The erythropoietin mutein of claim 2 wherein the amino acid at  
10   position 146 is replaced with the amino acid alanine.

11.   The erythropoietin mutein of claim 2 wherein the amino acid at  
position 147 is replaced with the amino acid alanine.

1        12.   The erythropoietin mutein of claim 2 wherein the amino acid at  
2    position 154 is replaced with the amino acid alanine.

1        13.   A DNA molecule encoding the erythropoietin mutein of  
2    claim 1.

1           14. A recombinant DNA construct comprising the DNA molecule  
2 of claim 13.

1           15. A vector comprising the DNA construct of claim 14 which is  
2 capable of expressing said erythropoietin mutein in a host cell.

1           16. A host cell comprising the DNA molecule of claim 13 capable  
2 of expressing the erythropoietin mutein encoded by said DNA molecule.

1           17. The host cell of claim 16 wherein said host cell is capable of  
2 glycosylating said erythropoietin mutein.

1           18. The host cell of claim 17 wherein said host cell is a mammalian  
2 cell.

1           19. The host cell of claim 18 wherein said host cell is selected from  
2 the group consisting of Chinese Hamster Ovary cells, Cos7 cells, Cos1 cells,  
3 baby hamster kidney cells, and CV1 cells.

1           20. A method of stimulating erythrocyte production in a subject,  
2 wherein said method comprises administering to said subject an efficacious  
3 amount of the erythropoietin mutein of claim 1.

1           21. A method of stimulating erythrocyte production in a subject,  
2 said method comprising administering to said subject an efficacious amount of  
3 an erythropoietin mutein comprising native erythropoietin with the amino acid  
4 residue at position 143 replaced by an alanine residue, wherein said efficacious  
5 amount is less than the corresponding amount of native erythropoietin.

1        22. A method of inducing the proliferation of a cell culture  
2 responsive to erythropoietin, wherein said method comprises contacting said  
3 cell culture with the erythropoietin mutein of claim 1.

1        23. A method of inducing the proliferation of a cell culture  
2 responsive to erythropoietin, wherein said method comprises contacting said  
3 cell culture with an efficacious amount of an erythropoietin mutein comprising  
4 native erythropoietin with the amino acid residue at position 143 replaced by  
5 an alanine residue, wherein said efficacious amount is less than the  
6 corresponding amount of native erythropoietin.

1        24. The method of claim 18 or 19 wherein said cell culture  
2 comprises cells selected from the group consisting of murine spleen cells,  
3 murine erythroleukemia cells, and human UT-7/Epo cells.

1        25. A method for obtaining erythropoietin muteins with enhanced  
2 biological activity relative to native erythropoietin, said method comprising the  
3 steps:

- 4            (a) preparing erythropoietin muteins comprising native erythropoietin  
5 with a single amino acid substitution, said substitution occurring at a position  
6 selected from the group consisting of positions 48-52, 151-160, and positions  
7 predicted to reside on the external surface of helices A and D;
- 8            (b) assaying the biological activity of the erythropoietin muteins from  
9 step (a) over a range of dosages; and
- 10          (c) identifying those erythropoietin muteins with enhanced biological  
11 activity relative to native erythropoietin.

		localization (bp)	<u>human</u> (1)	<u>mouse</u> (2)
IV1	5'	TCAAGTTGGCGAGAAGTGGATGCC	3'	984-1009
		AAGA(T/G)GTACCTCTCCAG(A/G)ACTGC	3'	994-1018
EX2R	5'	CIGCTCCACTCCGAACA(C/A)TCAC	3'	1296-1318
EX5	5'	CTGGAGTGTCCATGGGACAG	3'	1302-1324
NCO1	5'			2651-2672
				3183-3204
				3415-3434
ATG	5'	AGCCGGAGATGGGGTGC	3'	615-634
				6333-652

1  
FIG.

5  
FIG.

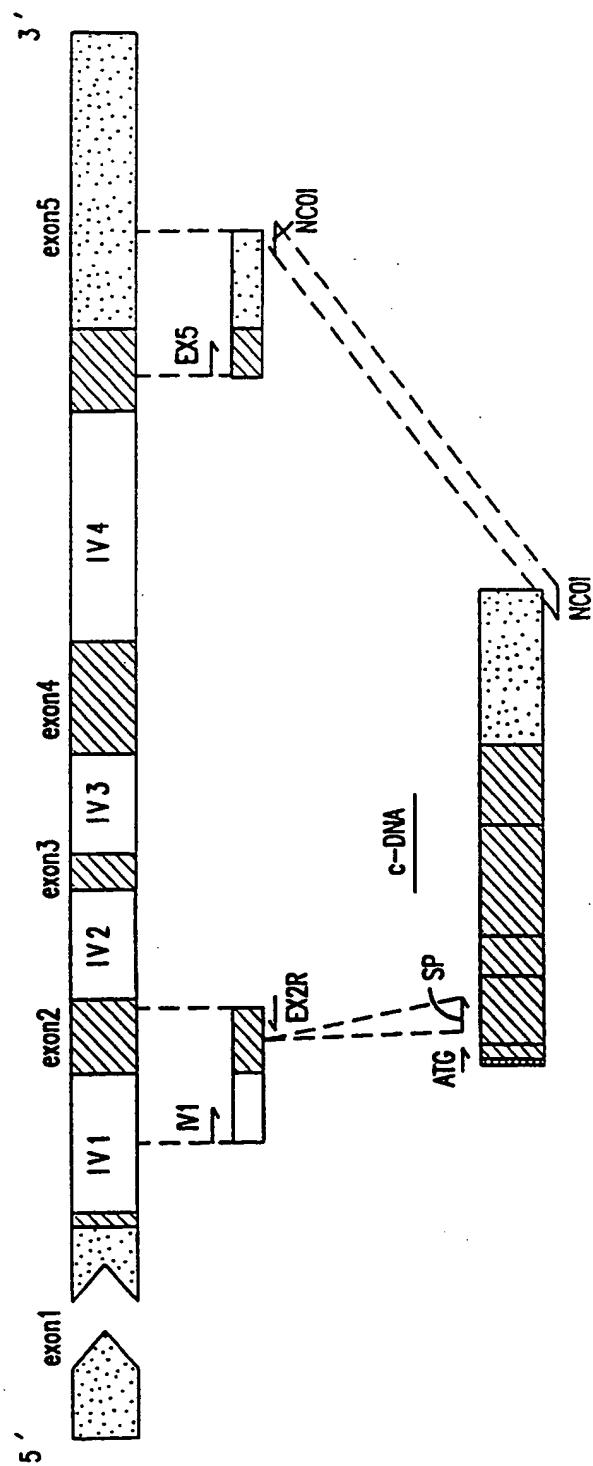


FIG. 2

FIG. 3

**SUBSTITUTE SHEET (RULE 28)**

FIG. 3A

**SUBSTITUTE SHEET (RULE 26)**

FIG. 4

CGTAAAGTC TGGCAGGCC TGGCCCTGCT GTCGGAAGCT GTCCCTGGGT CCCAGGCCCT GTTGGTCAAC TCTCCCAGC CGTGGGAGCC  
 TGTAAAGTC TGGCAGGCC TGGCCCTGCT CTAGAAGCT GTCCAGGGT GTCTGGGG CCCAGGGGGT GTTGGCAAC TCTCCCAGC CTTTCGAGCC  
 CATAGAAGT TGGCAAGGCC TGTCCCTGCT CTAGAAGCC ATCCCTGAGG CCCAGGCCCT GCTAGCCAAT TCCCTCCAGC CACCAAGAC  
 TGTAAAGT TGGCAAGGCC TGTCCCTGCT CTAGAAGCC ATCCCTGAGG CCCAGGCCCT GCAGGCCAT TCCCTCCAGC CACCAAGAG  
 TCTGGAAGTC TGGCAGGCC TGGCCCTGCT CTAGAAGCT ATCTTGGG CCCAGGCCCT ACCGGCAAC GCACTCCCAGC CATGGGAGC  
 CATGGAGTC TGGCAGGCC TGTCCCTGCT CTAGAAGCC ATCCCTGAGG CCCAGGCCCT GTTGGCAAC TCCCTCCAGC CATCTGAGC  
 TGTGGAAGTC TGGCAGGCC TGGCCCTGCT CTAGAAGCC ATCCCTGAGG CCCAGGCCCT CCTGGCAAC TCCCTCCAGC CATCTGAGAC

350

CCTGGAGCT CAGTGGATA AAGCGTCAAG TGGCCTTCGC AGCCCTACCA CTCGGCTTG GGCTCTGGGA CCCAGAAAAG AAGCCATCTC  
 CCTGGAGCT CACATGGATA AAGCCATCAAG TGGCCTTCGC AGCATACCA CTCGGCTTG GGGCTGGGA GGGCAAG—G AAGCCATCTC  
 CCTTAGCTT CATAAGACA AAGCCATCAAG TGGTCTACGT AGCCCTACTT CACIGCTTCC GGTACTGGGA GCTACAGAAGC AATTGATGTC  
 CCTTAGCTT CATAAGACA AAGCCATCAAG TGGGCTACGT AGCCCTACTT CACTGGCTTG CGTGGCTGGGA GCTACAGAAGG AATTGATGTC  
 CCTGGGGTTG CACCTGGACA AAGCTGTCAAG GGGCTCCGC AGCTCACTT CCCTGGCTTG GGGCTGGGA GGGCAAGAAG AAGCCATCCC  
 CCTGGAGCT CATGGACA AAGCTGTCAAG GGGCTGGGC AGCCCTACCT CCCTGGCTTG GGGCTGGGA GGGCAAGG AAGCCATCCC  
 CCTGGAGCT CATGTGGACA AAGCTGTCAAG CAGGCGTCAAG AGGCGCTGGC AGGCGCTACCT CCCTGGCTTG GGGCAAGGG AAGCCACCTC

400

CCTGGAGCT GGGGCC—I CAGCTG—I TCCACTCCGA AGAAATCCGA CTCGACACTT CTCGAAACTT CTCGAGCTC TTCCCAAAIT  
 CCTGGAGCT GGGGCC—I CAGCTG—I TCCACTCCGA ACCATCTACTG CTGACACTT CTGCAAACCTC CTGCGAGTCT ACTCCAAIT  
 CCTGGAGCT ACCACC—I CACCTG—I TCCACTCCGA ACACCTCACAG TGGAATCTT CTGCAAACCTC TTCCGGGTCI ACTCCAACT  
 CCTGGAGCT GCCACC—I AAGCGG—I TCCACTCCGA ACACCTCACAG CGGATACCTT CTGCAAACCTC TTCCGGGTCI ACTCCAACT  
 CCTGGAGCT GGGCAAG—C CCCACTCCGA ATATTCACTG TTGAIGCTT GICCAAGCTC TTCCGAATCT ACTCCAAIT  
 CCTGGAGCT GCAACCCCT CCTCTGCCAC CCCACTCCGA ACATTTGCTG TTGAATCTT GICCAAACCTT TTCCGGAACTT ACTCCAAIT  
 CCTGGAGCT GCAACC—I CTGCTG—I TCCACTCCGA ACATTCACCT CGATACTT GTGCAAACCTT TTCCGAATCT ACTCCAACT

500

**FIG. 4A**

550	CCTCGGGGA AAGCTGAAAC TG TACACAGG GGAGCCCTGC AGGACAGGG ACAGATGACC CCTCCGGGA AACCTGAAAC TG TACACGGG CGAGGCCCTGC AGGAGGGG ACAGATGACC CCCTCGGGGA AAACCTGAAAC TG TACACGGG AGAGCTTGC AGGAGGGG ACAGGTGACA CCTCCGGGG AACACTGAAAC TG TACACGGG GGAGCCCTGC AGGAGGGG ACAGGTGACA CCTGAGGGGA AACCTGACCC TG TACACAGG GGAGGCCCTGC AGGAGGGG ACAGGTGACC CCTTGCGGGGA AACCTGACCC TG TACACTGG AGAGCCCTGC AGGAGAAGGG ACAGGTGACT	600	ACAGTGGGCA TATCCACCAAC ACC-TGGGCA TATCCACCAAC AGC TGGGCA CATTCCACCAAC ACC-TGGGCA TATCCACCAAC ACC-CGGGTC ACC-TGGGTC ACC-TGGGTC ACC-TGGGTC TCC TGGTACA ACAGGTGACA TCC TGGTACA ACAGGTGACA TGC ——C ACT-GCCGTG TGC ——C ACT-GCCGTG TACCCGCCAA ACCCGGCA CGTCCATCAC CGTCCACCAAC ACCGGGGCA TGTTCCACAC
650	CCTCCCTTACCC AACATTCCTT GTGCCACACC CTCCCCCG— CTCCCCTTACCC AACACTGGCT GTGCCACACC CTCCCC— CTTGGCTCCCC GTCACTGTGT CATGCCAACCC CTCC— CTGGCTTACCC GTCACTGTGT CAGGCCAACCC CTCC— CTGGCTTACCC ATCACTGGCT -AGGCCCATGCC CTTCCAGGCC GCCACTCCC— TTGGCTTACCC ACCACTGGCT GTGCCATGCC TTCT-CCACC ACCACTCCC— CTCACTTACCC ACCATTCACCC ACCACTGGCT GTGCCACGCC	700	CGA—GGGG— CGA—GGGG— CGA—AT CGA—GGGG— CAAAC—TCAT CAAAC—GGG —TCAT-TAC CTTCTTACCA AAACCGGGCT CAACCGGGCT TGTGTGTAC CTTCTTACCG A—CCCC-TGT CGAAGCAGGG —CCACCG CTCAGT A—CCCCC-GC TGA—GGGG —CCATTCAG CTCAGGCCCA C—CCCC-TGT C—GGGG TG—ATCAG CTCAGGCCCA
742	GCCTGTCCCC TGGCACACTCC AG GC —— GT —— GC —— GC —— GC —— GC —— GC —— GC ——		

FIG. 4B

<b>Human</b> <b>Macaca fascicularis</b> <b>Macaca mulatta</b> <b>Mouse</b> <b>Rat</b> <b>Sheep</b> <b>Pig</b> <b>Cat</b>	<b>A</b> 1 APPRLICDOSRVL 10 APPRLICDOSRVL 20 APPRLICDOSRVL 30 APPRLICDOSRVL 40 APPRLICDOSRVL APPRLICDOSRVL APPRLICDOSRVL APPRLICDOSRVL APPRLICDOSRVL APPRLICDOSRVL APPRLICDOSRVL APPRLICDOSRVL	<b>B</b> 10 QAVEVWQG 20 QAVEVWQG 30 QAVEVWQG 40 QAVEVWQG 50 QAVEVWQG 60 QAVEVWQG 70 QAVEVWQG 80 QAVEVWQG 90 QAVEVWQG 100 QAVEVWQG 110 QAVEVWQG	<b>C</b> 10 QLHVDKAVSS 20 QLHVDKAVSS 30 QLHVDKAVSS 40 QLHVDKAVSS 50 QLHVDKAVSS 60 QLHVDKAVSS 70 QLHVDKAVSS 80 QLHVDKAVSS 90 QLHVDKAVSS 100 QLHVDKAVSS 110 QLHVDKAVSS	<b>D</b> 10 QLHVDKAVSS 20 QLHVDKAVSS 30 QLHVDKAVSS 40 QLHVDKAVSS 50 QLHVDKAVSS 60 QLHVDKAVSS 70 QLHVDKAVSS 80 QLHVDKAVSS 90 QLHVDKAVSS 100 QLHVDKAVSS 110 QLHVDKAVSS	<b>E</b> 10 KEPSPDAASAA 20 -EAIISLPDAASAA 30 -EAIISLPDAASAA 40 -KELMSPPDATPPA 50 -KELMSPPDATPPA 60 KEAIPLPDATPSSA 70 KEAIPLPDATPSSA 80 KEATSLPEAT-SAA
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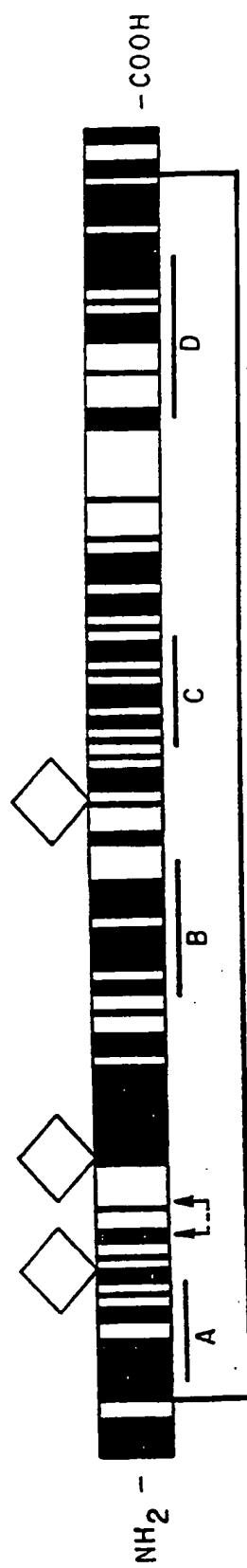


FIG. 6A

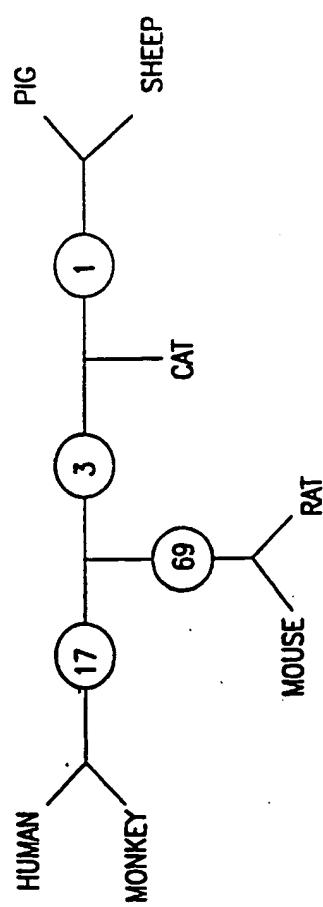


FIG. 7

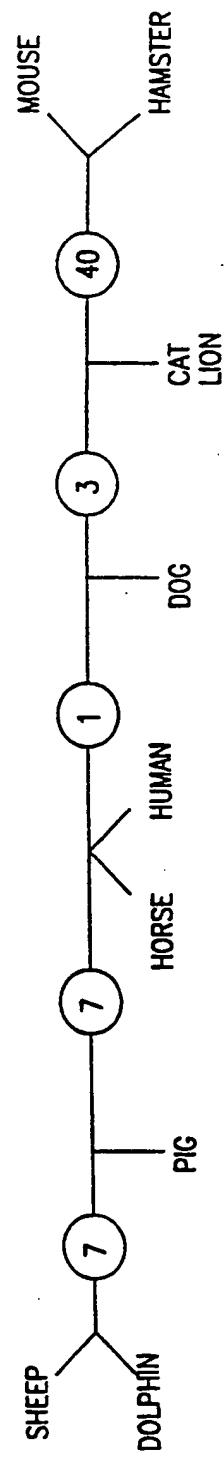


FIG. 8

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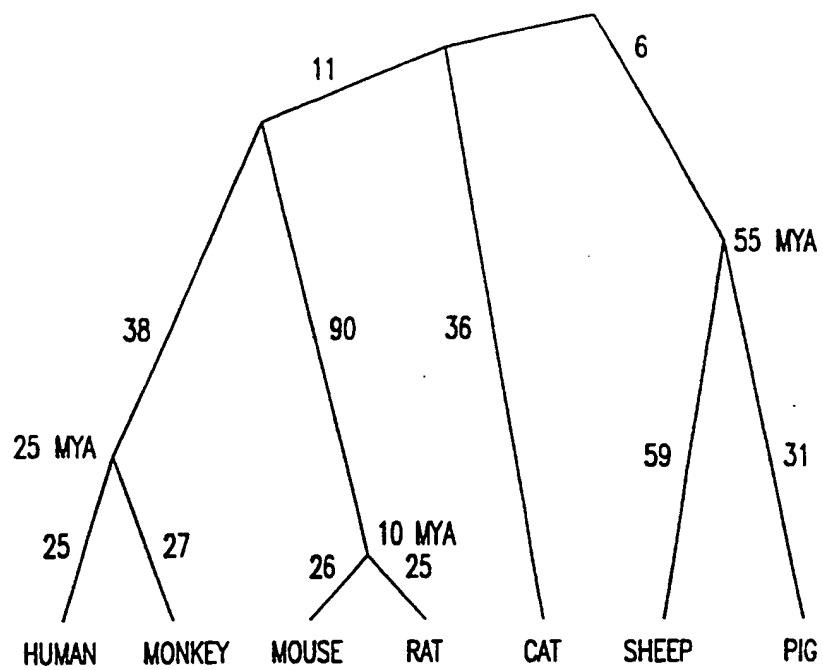


FIG.7A

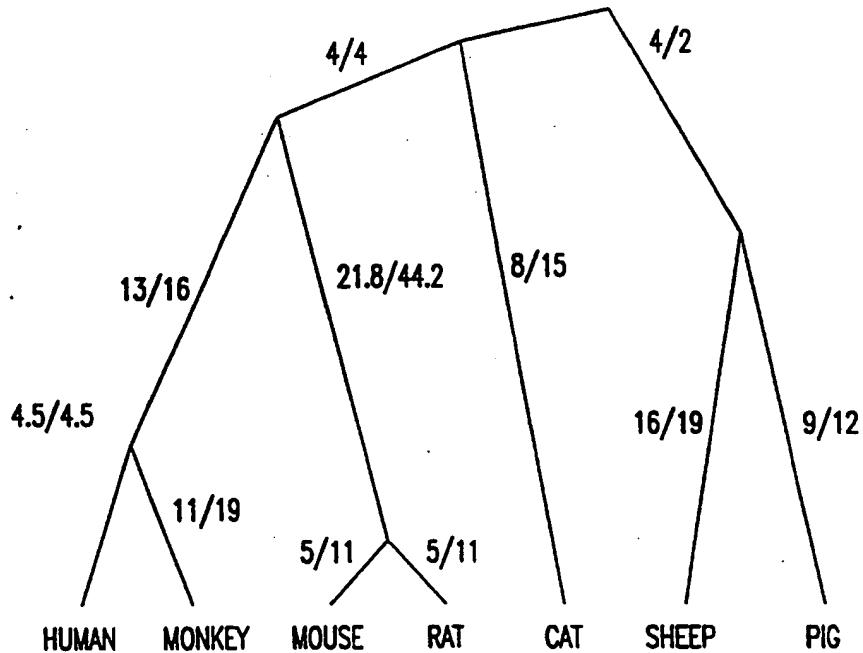


FIG.7B

SUBSTITUTE SHEET (RULE 26)

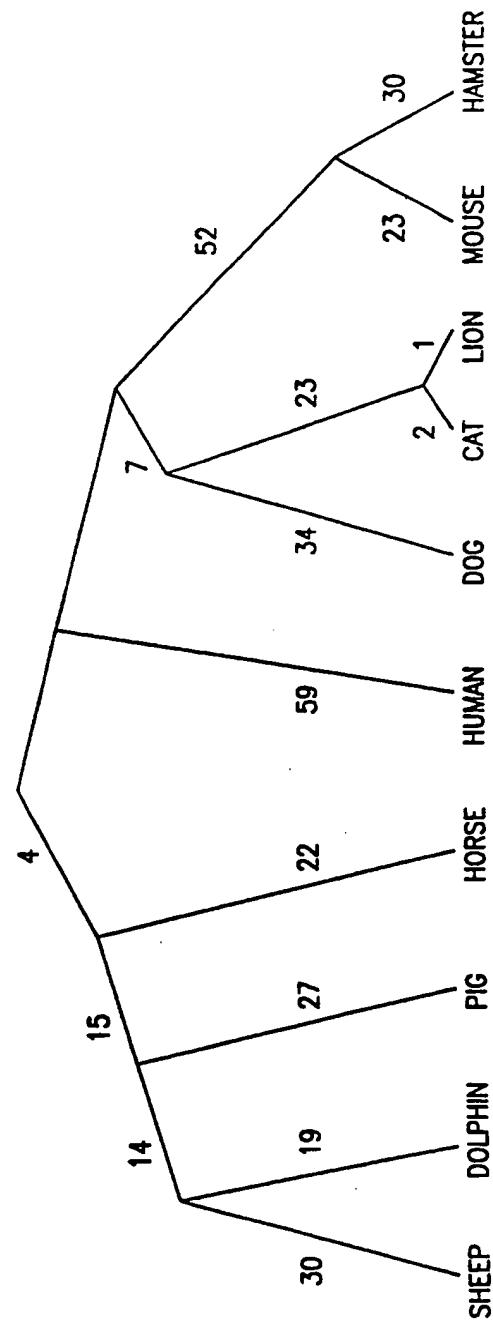
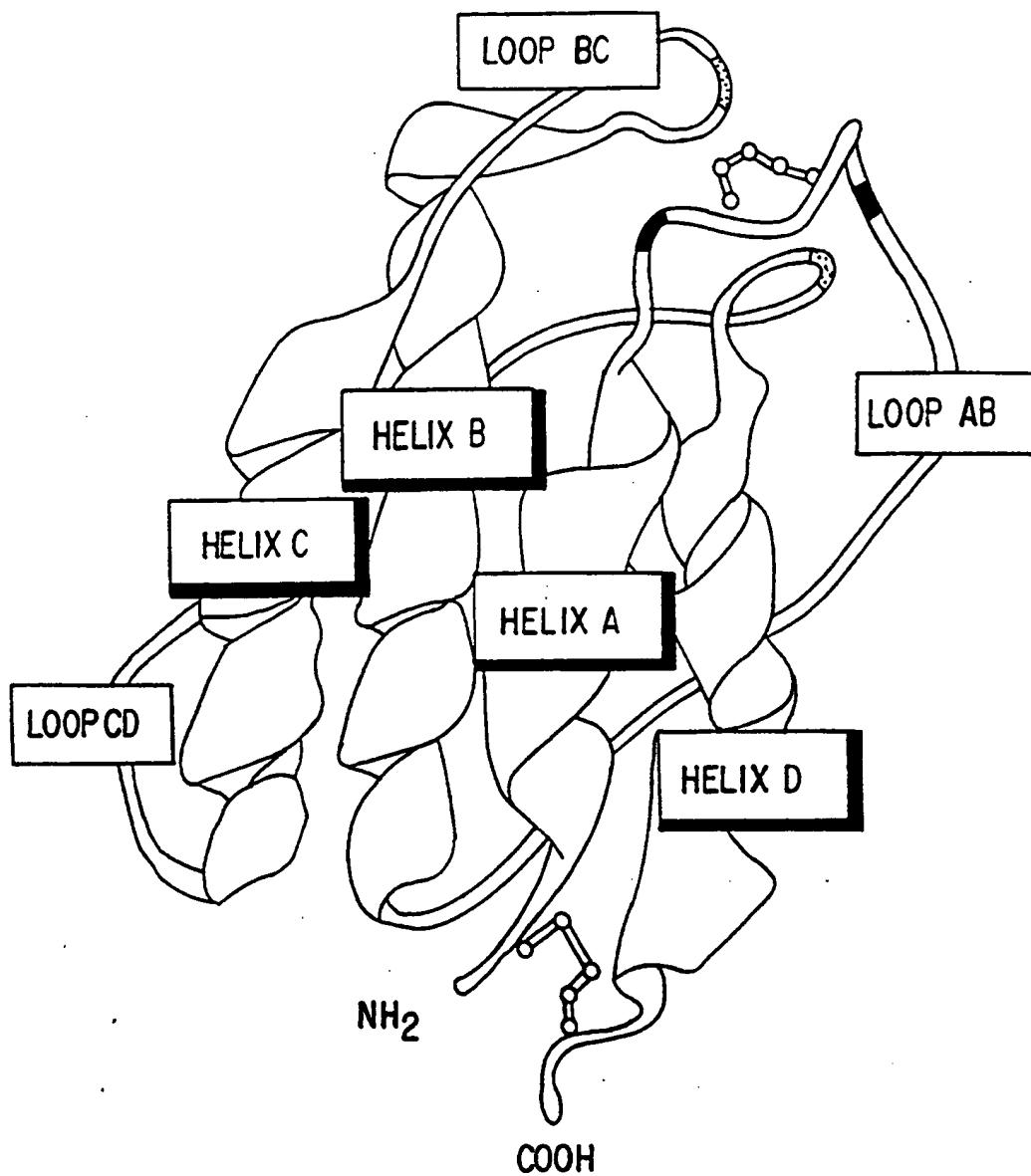


FIG. 8A



■ N-GLYCOSYLATION SITES  
▨ O-GLYCOSYLATION SITE

FIG. 9

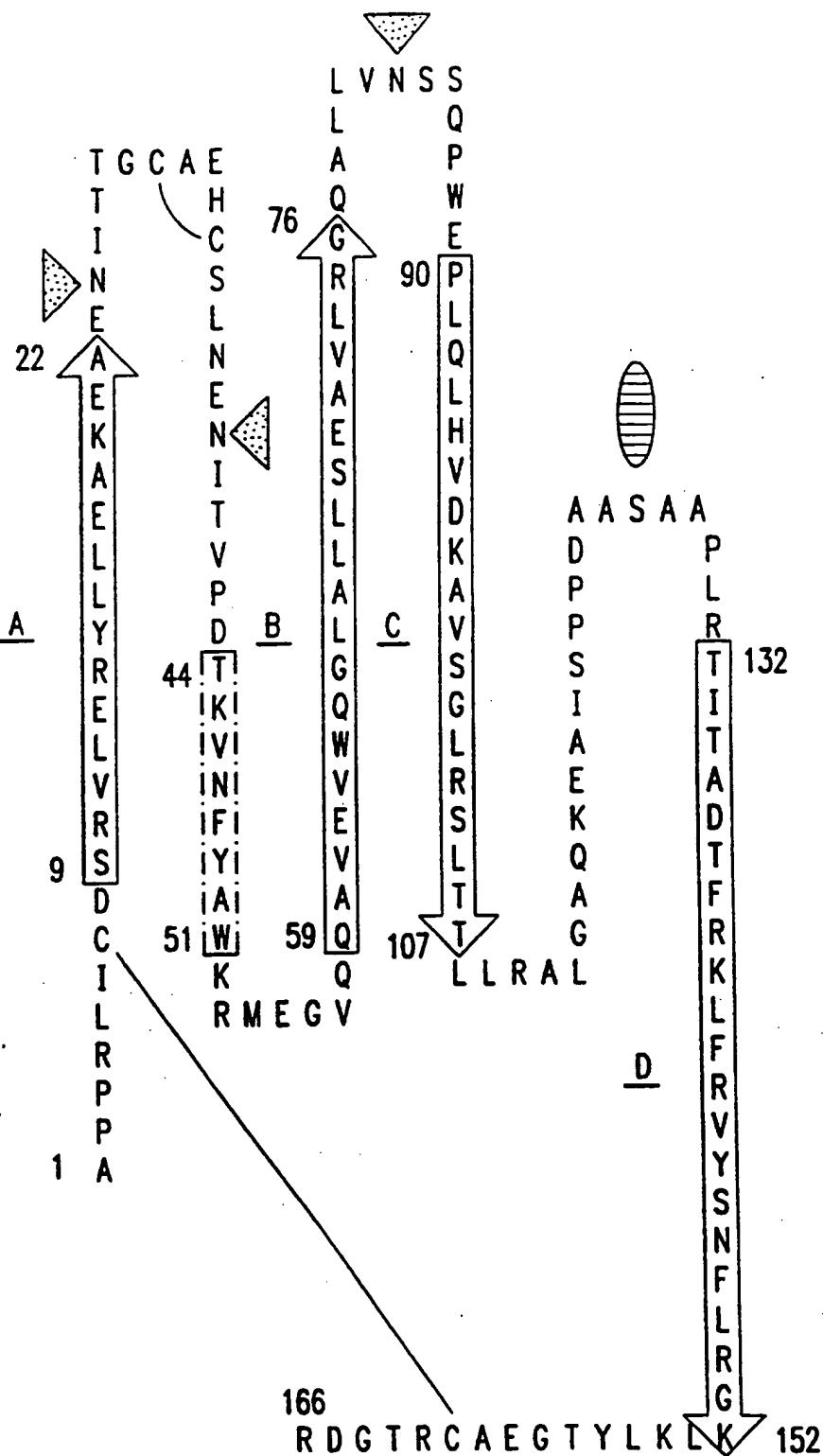


FIG.9A

SUBSTITUTE SHEET (RULE 26)

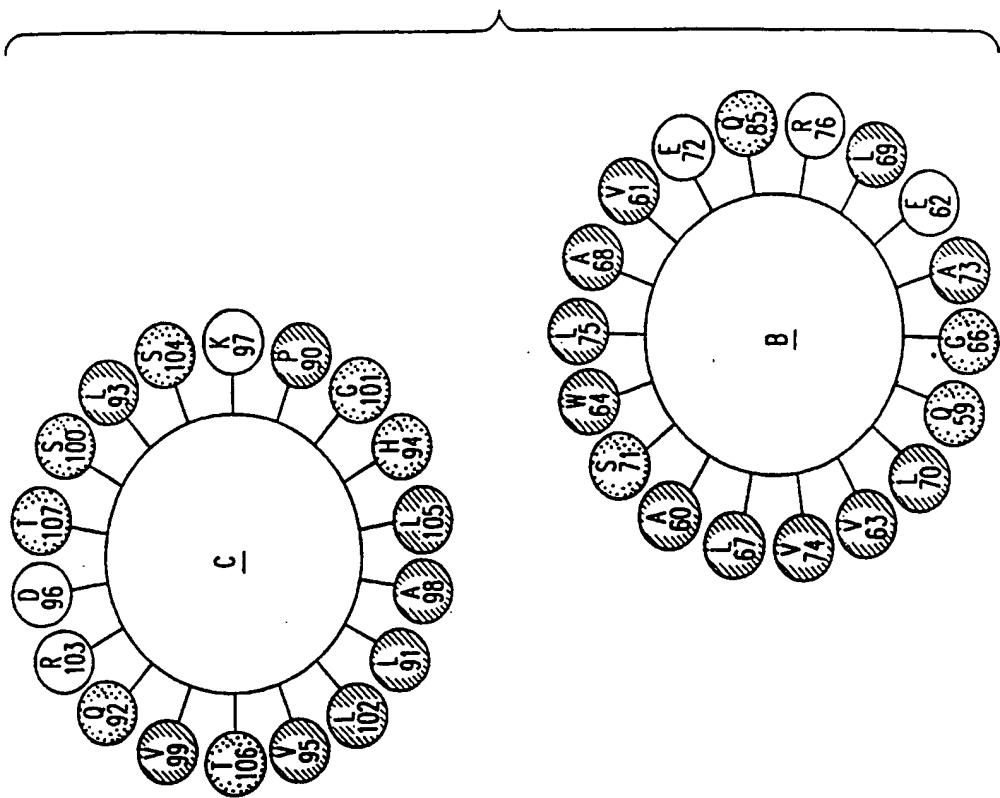
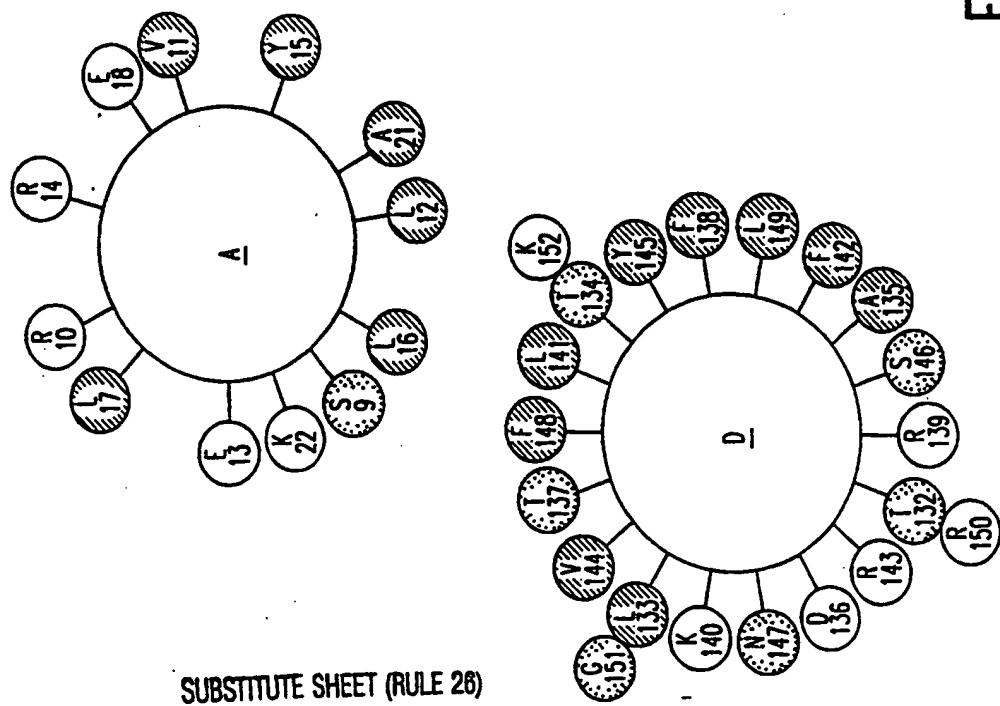


FIG. 9B



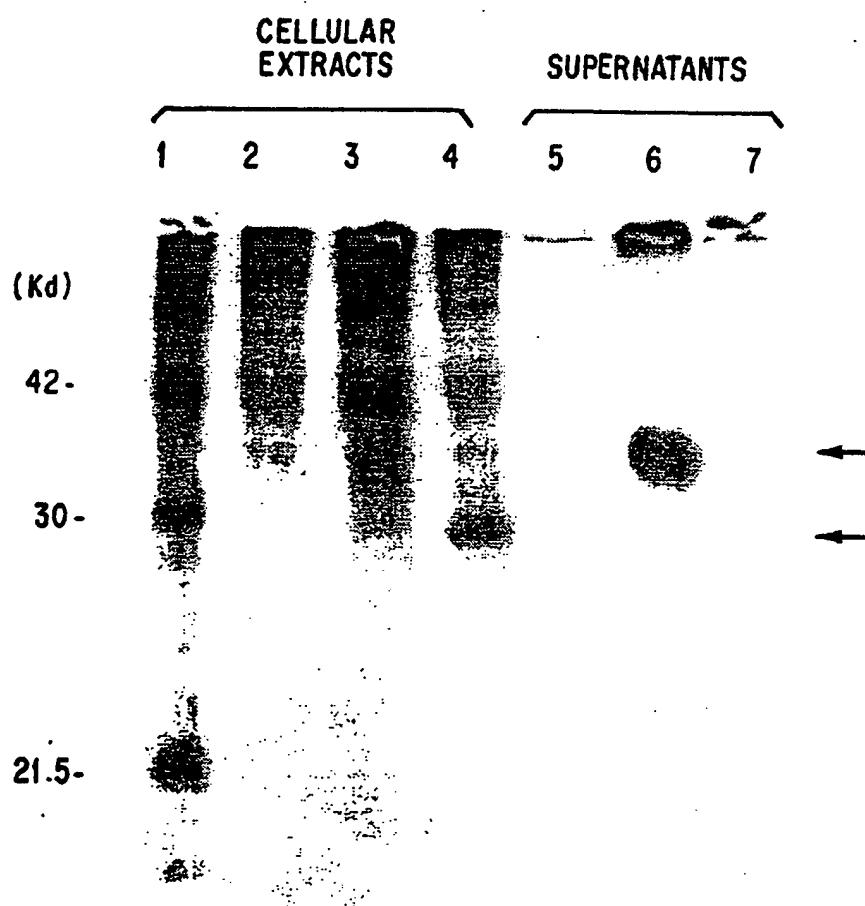
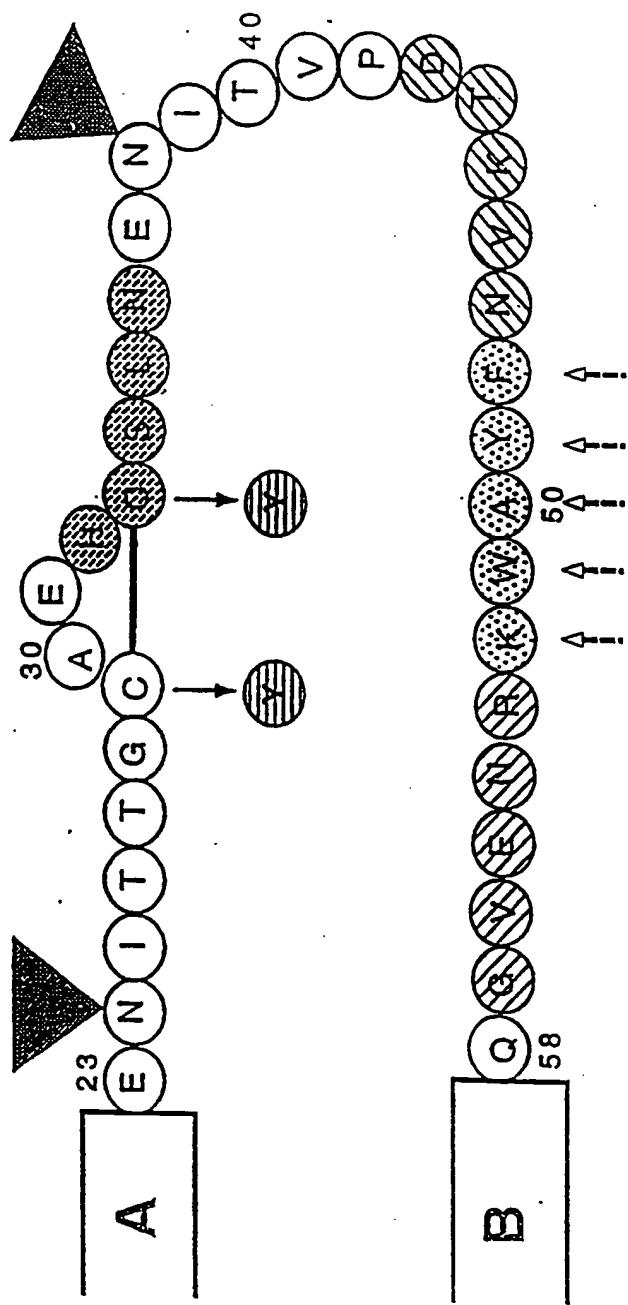


FIG. 10

LOOP A.B.

- FIGURE 11 -

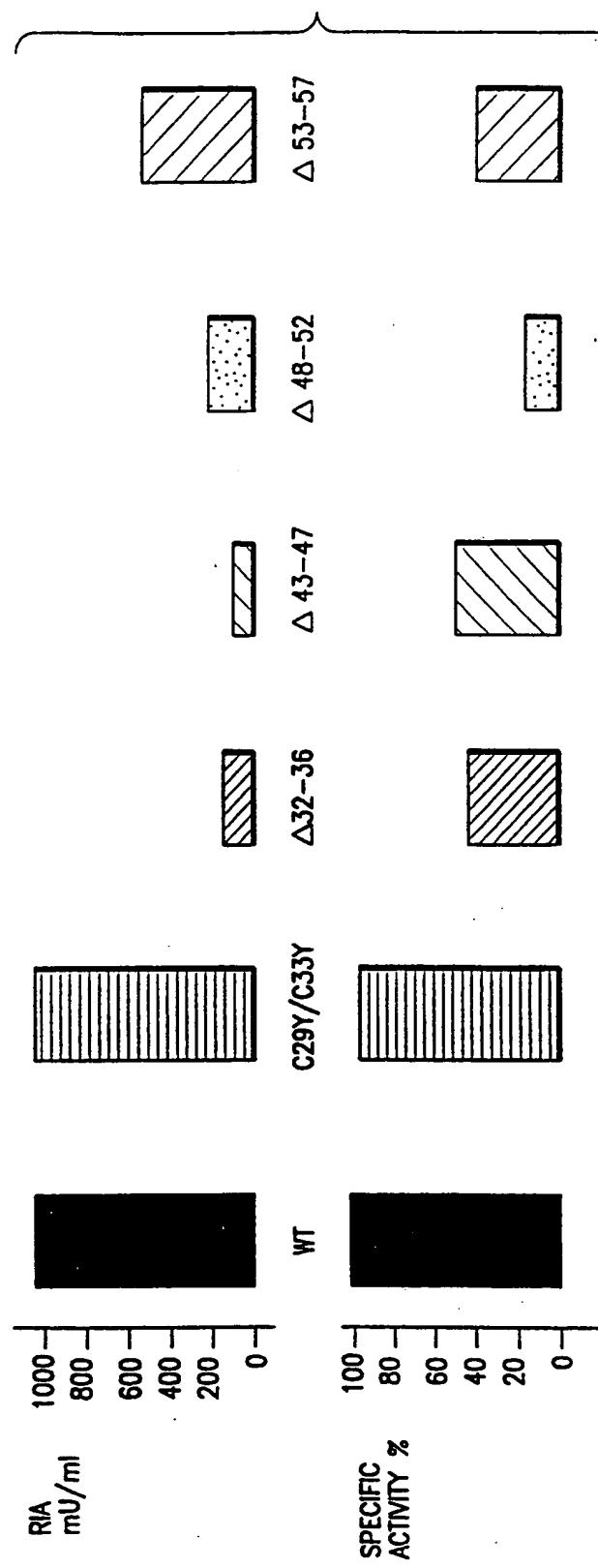
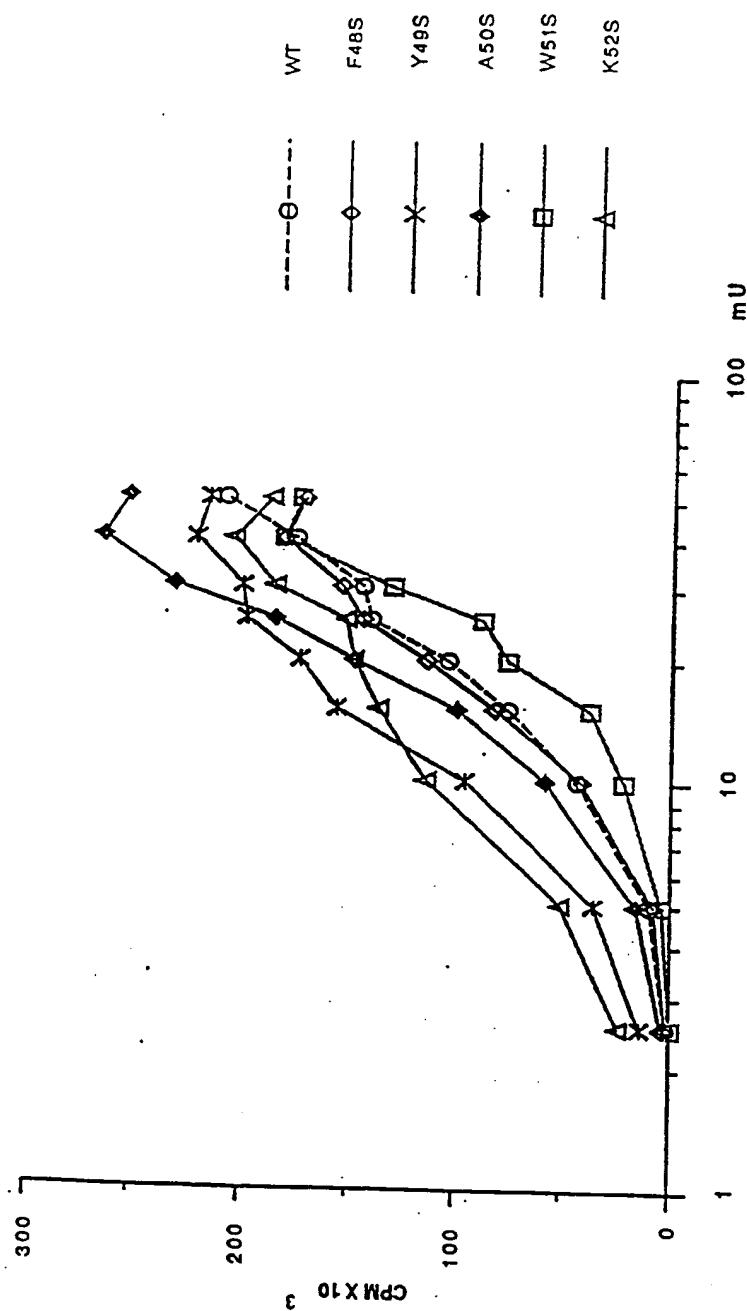
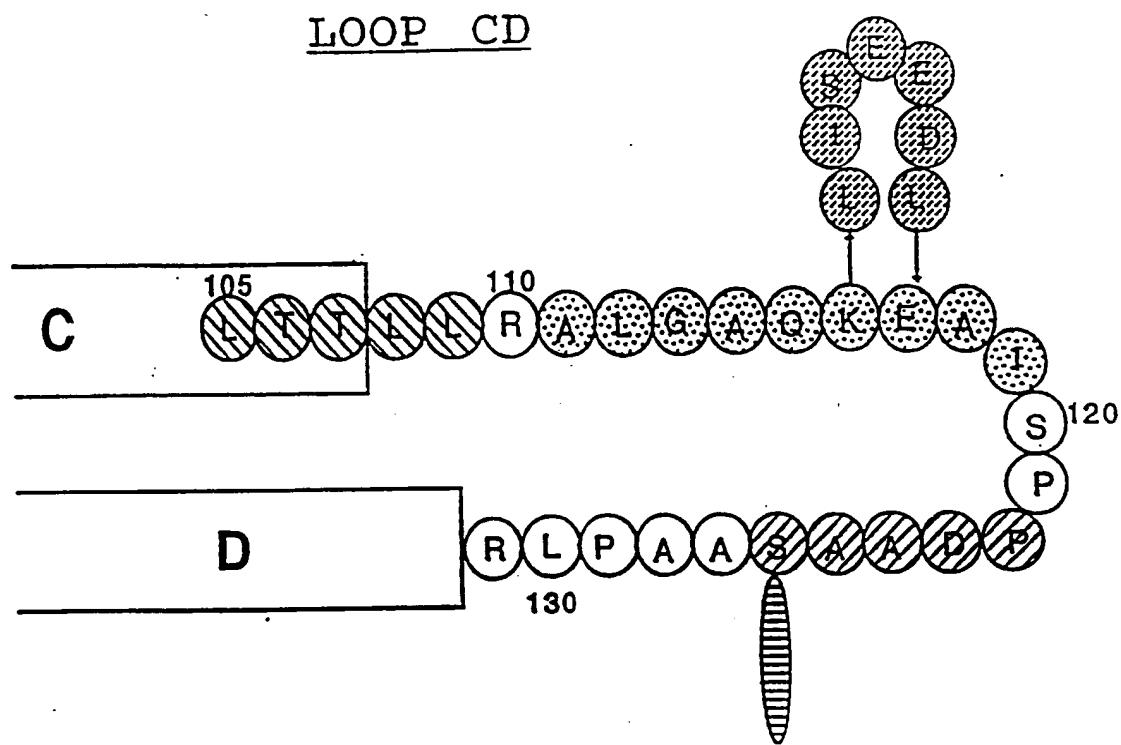


FIG. 11A



- FIGURE 11B -



- FIGURE 12 -

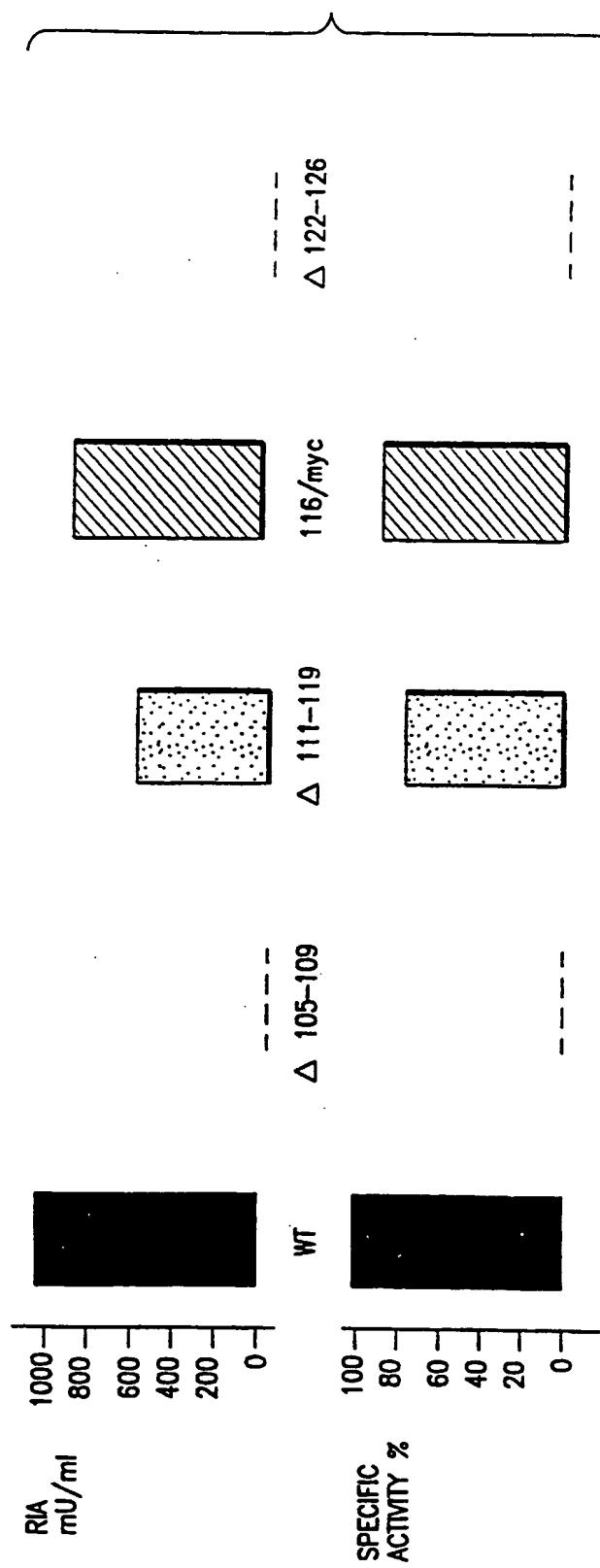


FIG. 12A

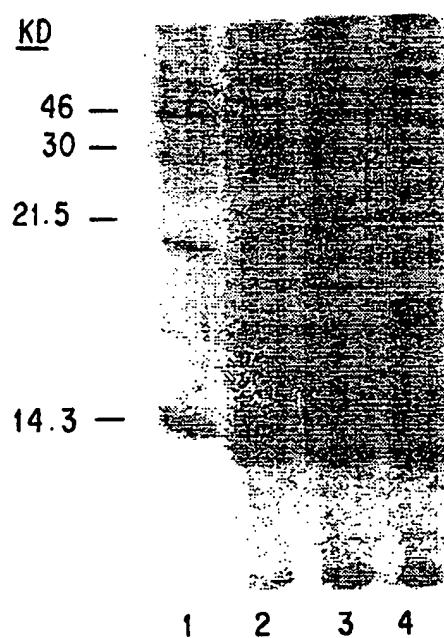
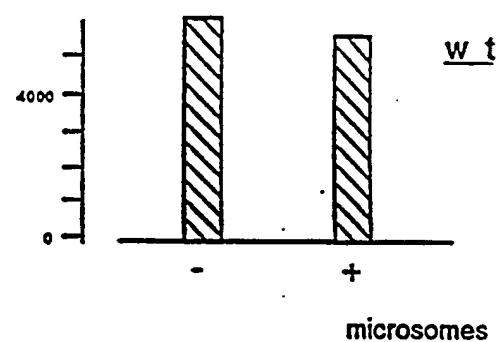


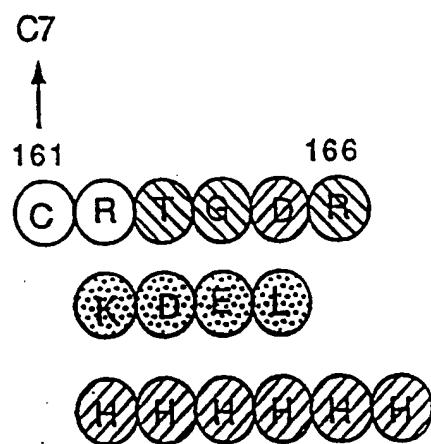
FIG.13

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- FIGURE 13A -

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C terminus.

- FIGURE 14 -

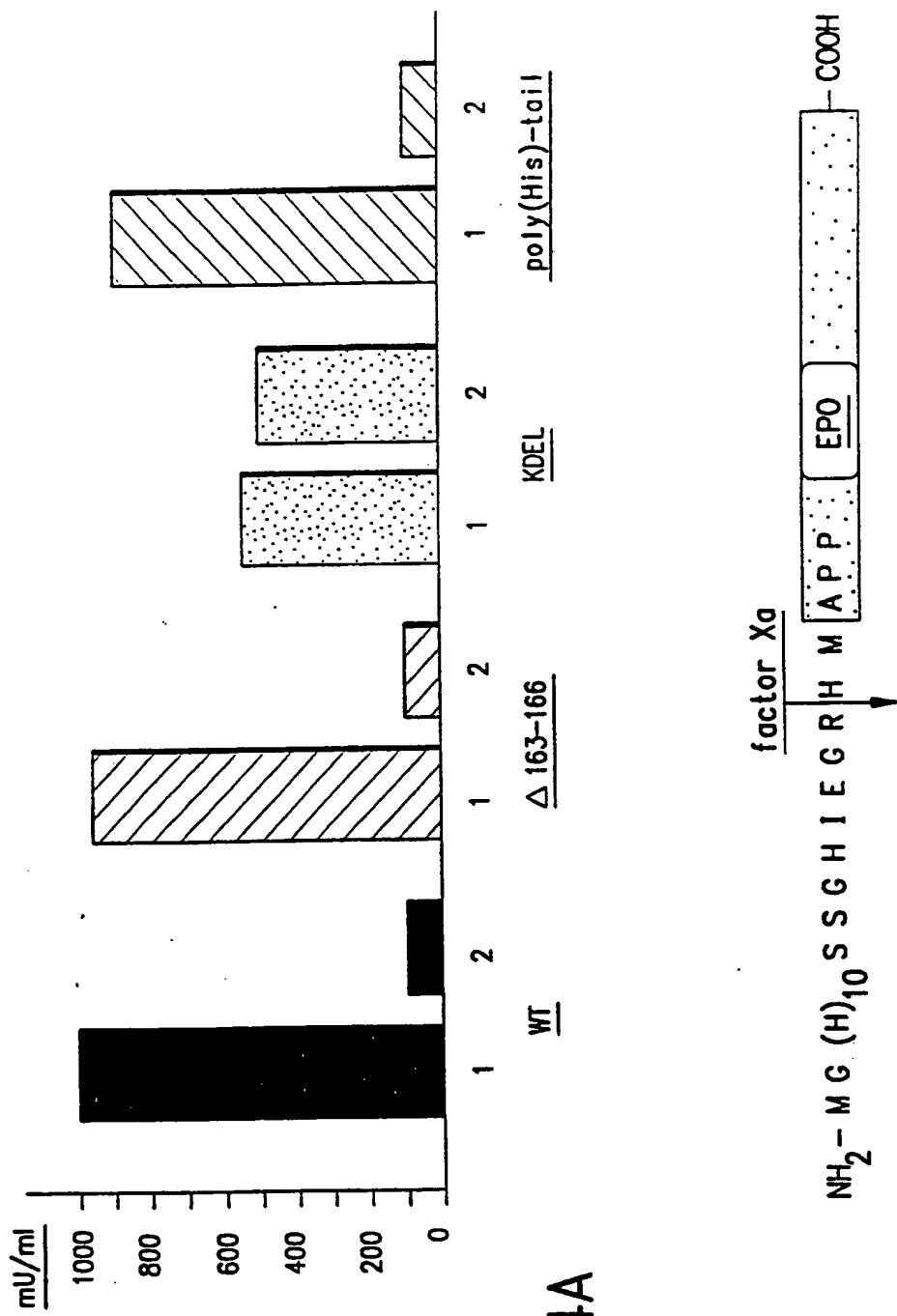


FIG. 14A  
SUBSTITUTE SHEET (RULE 26)

FIG. 15

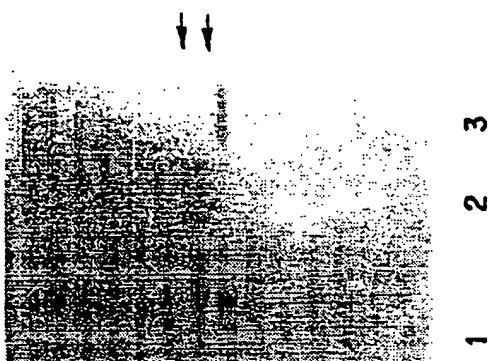


FIG. 15C

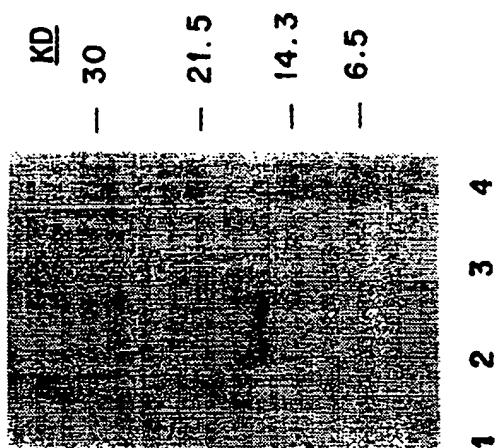


FIG. 15B

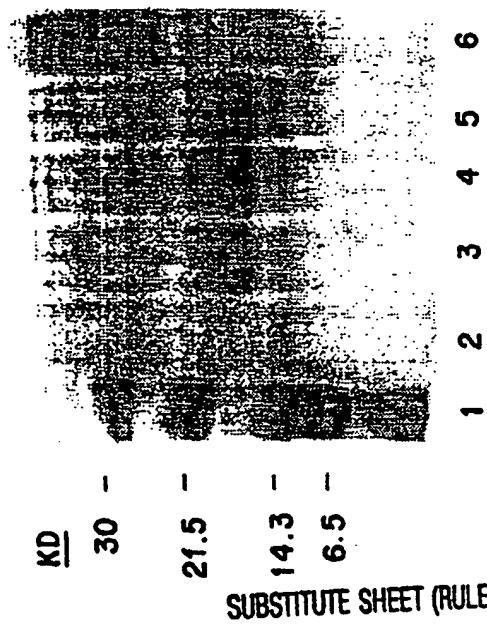


FIG. 15A

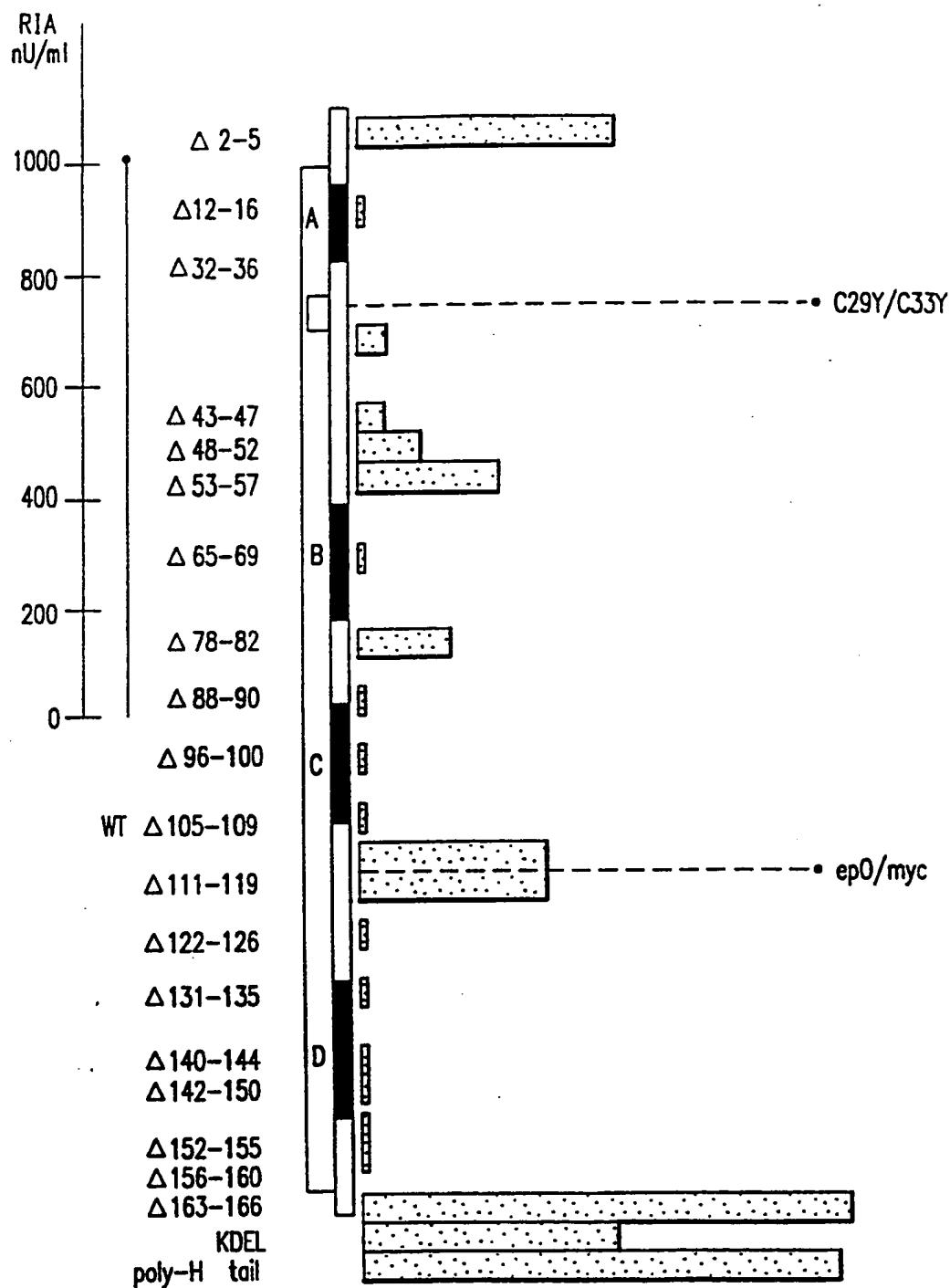
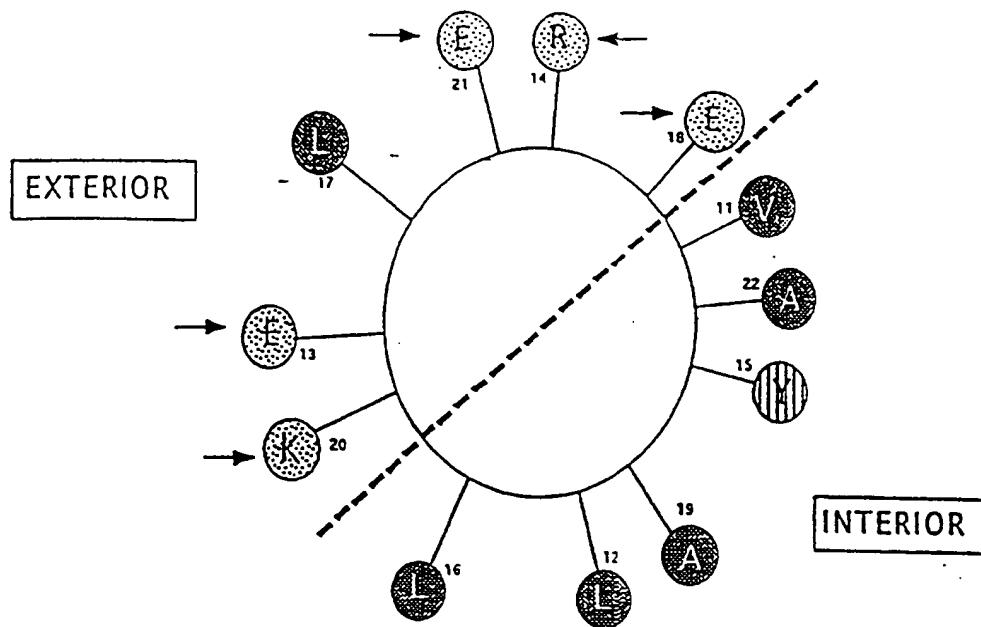
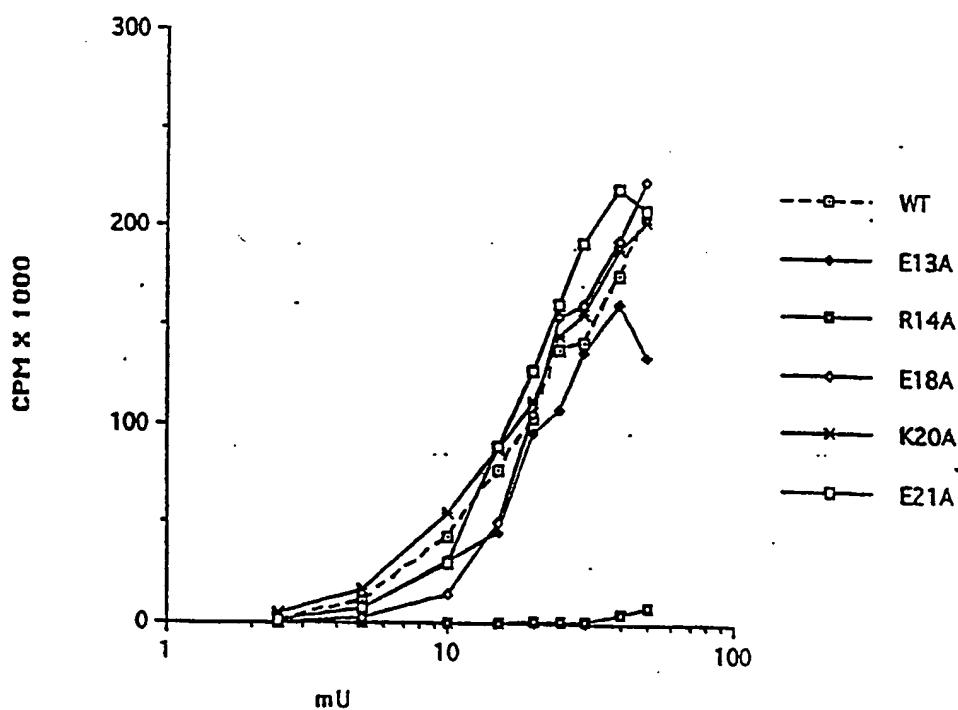


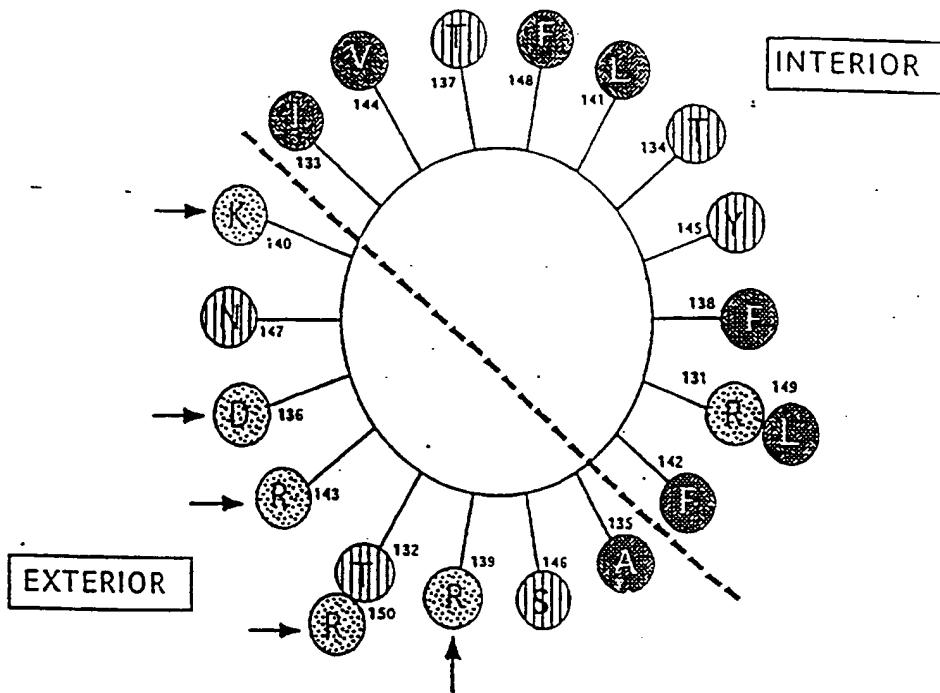
FIG.16

A HELIX

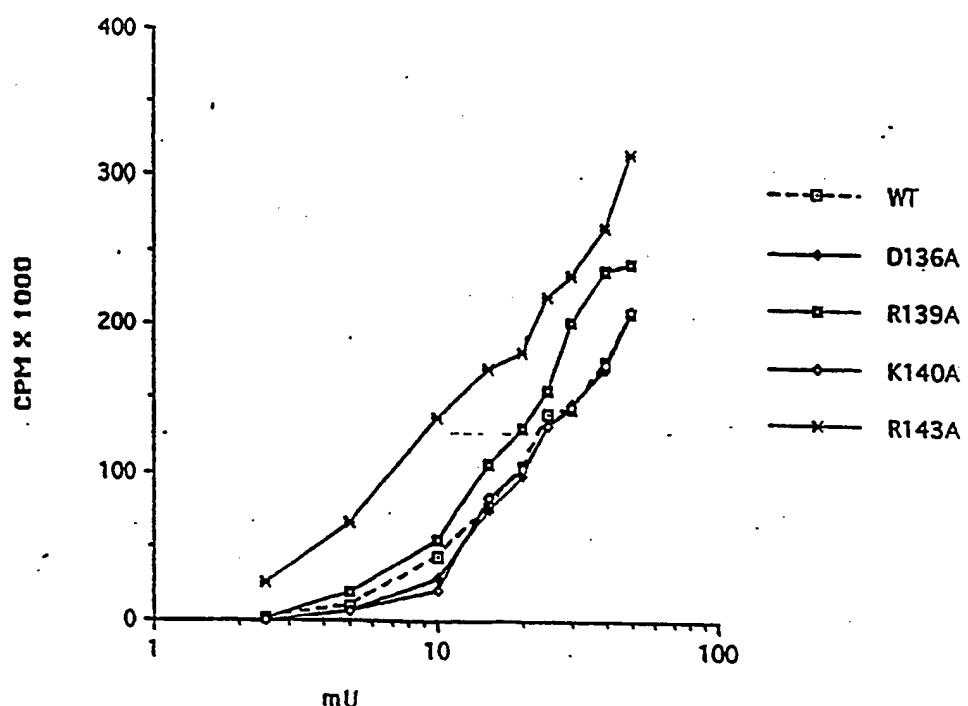
HCD 57



- FIGURE 17 -

D HELIX

HCD 57



- FIGURE 18 -

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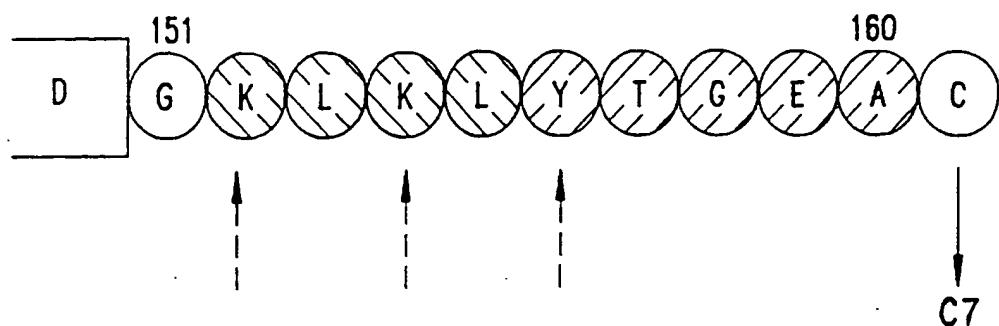


FIG. 19

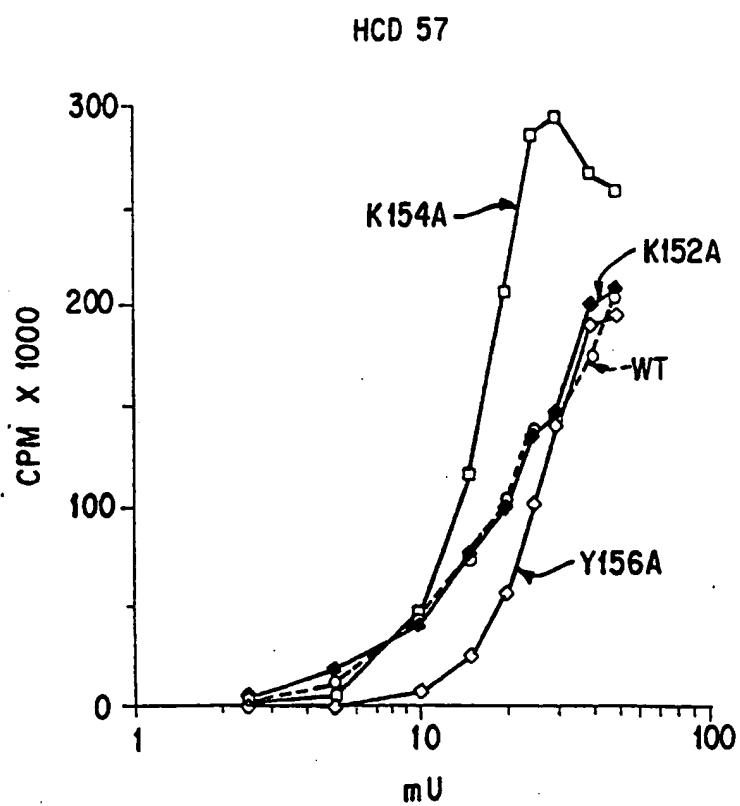


FIG. 19A SUBSTITUTE SHEET (RULE 26)

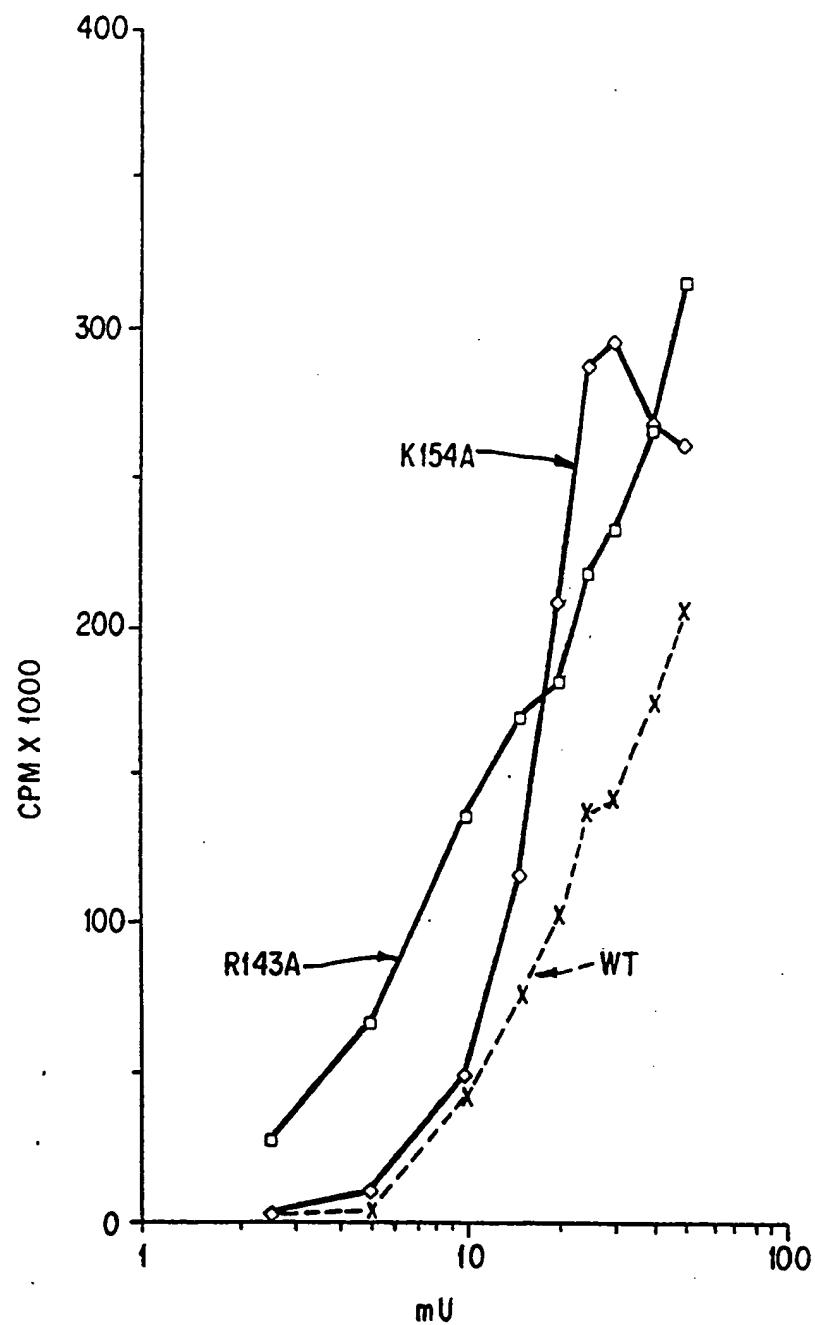


FIG. 20

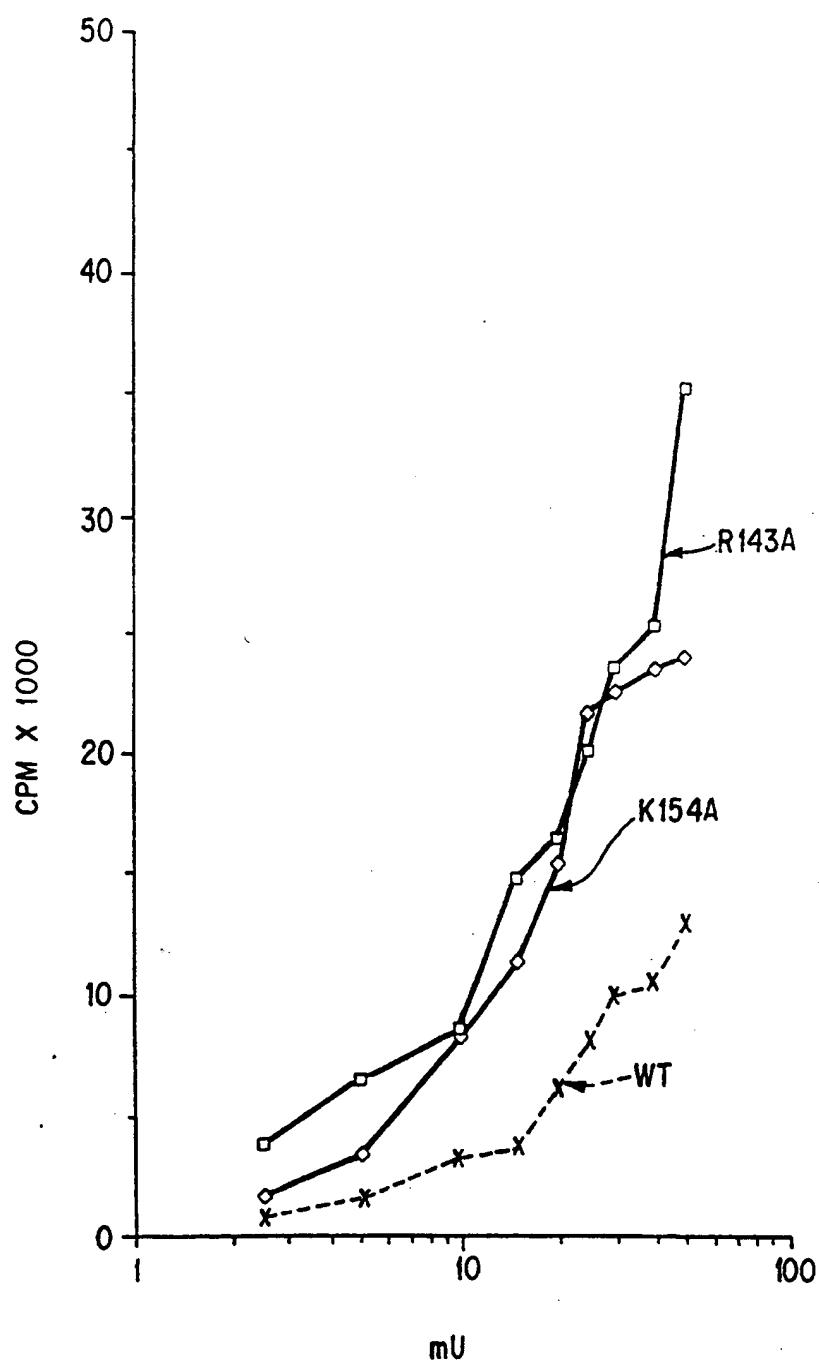


FIG. 21

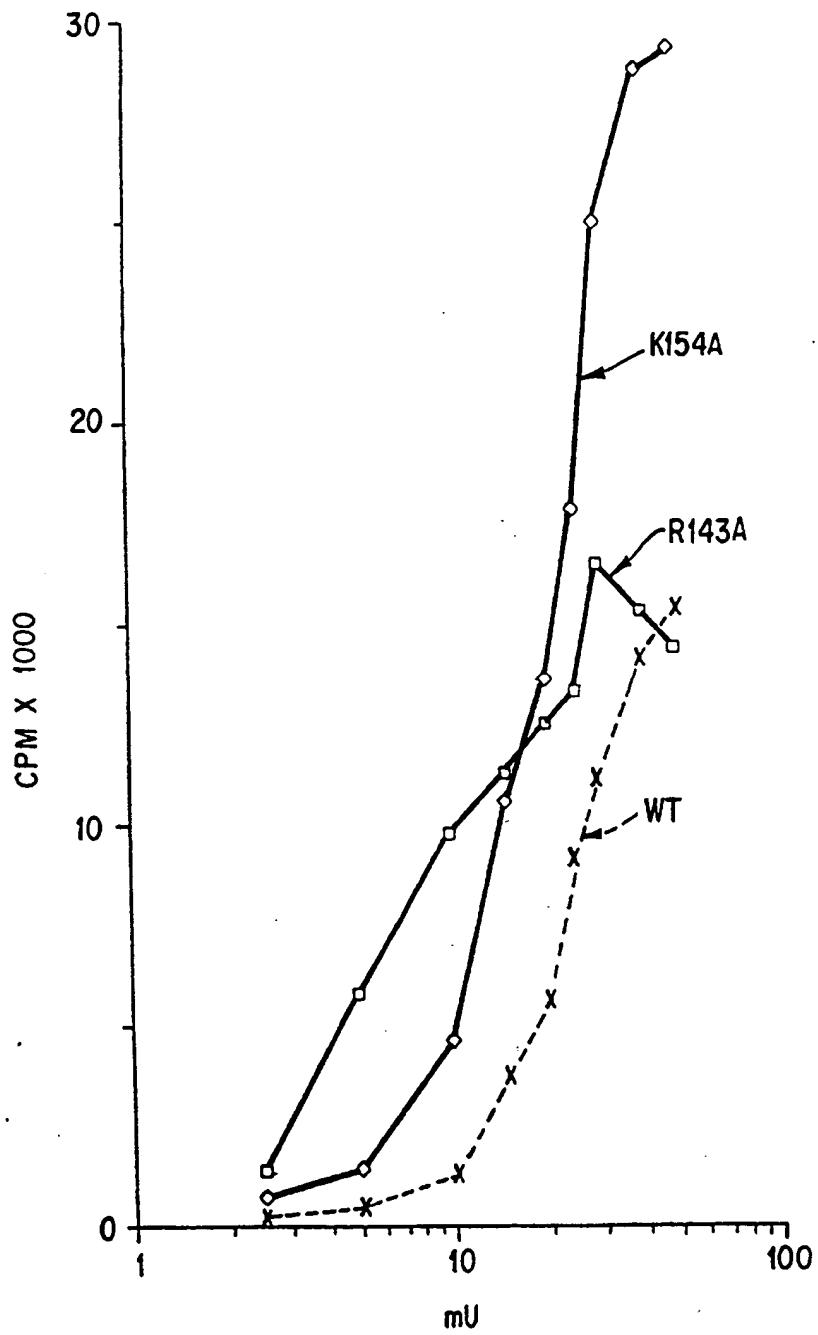


FIG. 22

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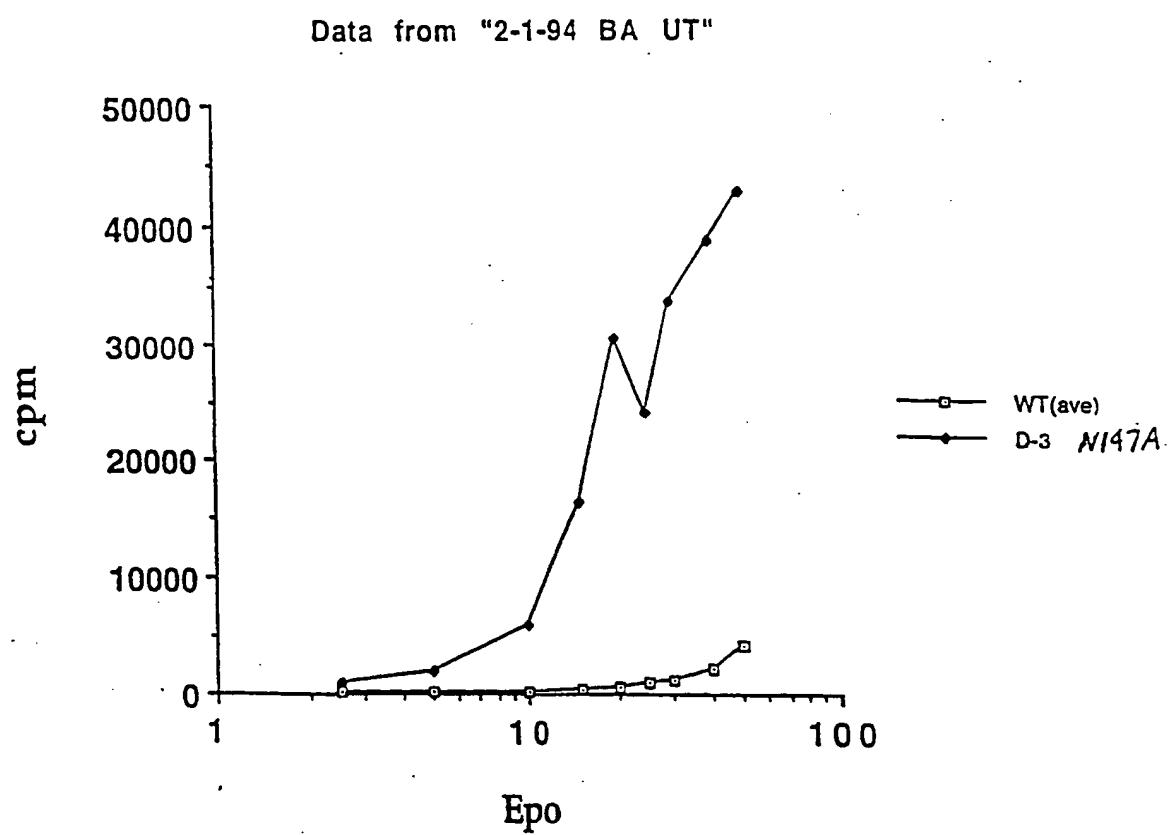


FIGURE 23

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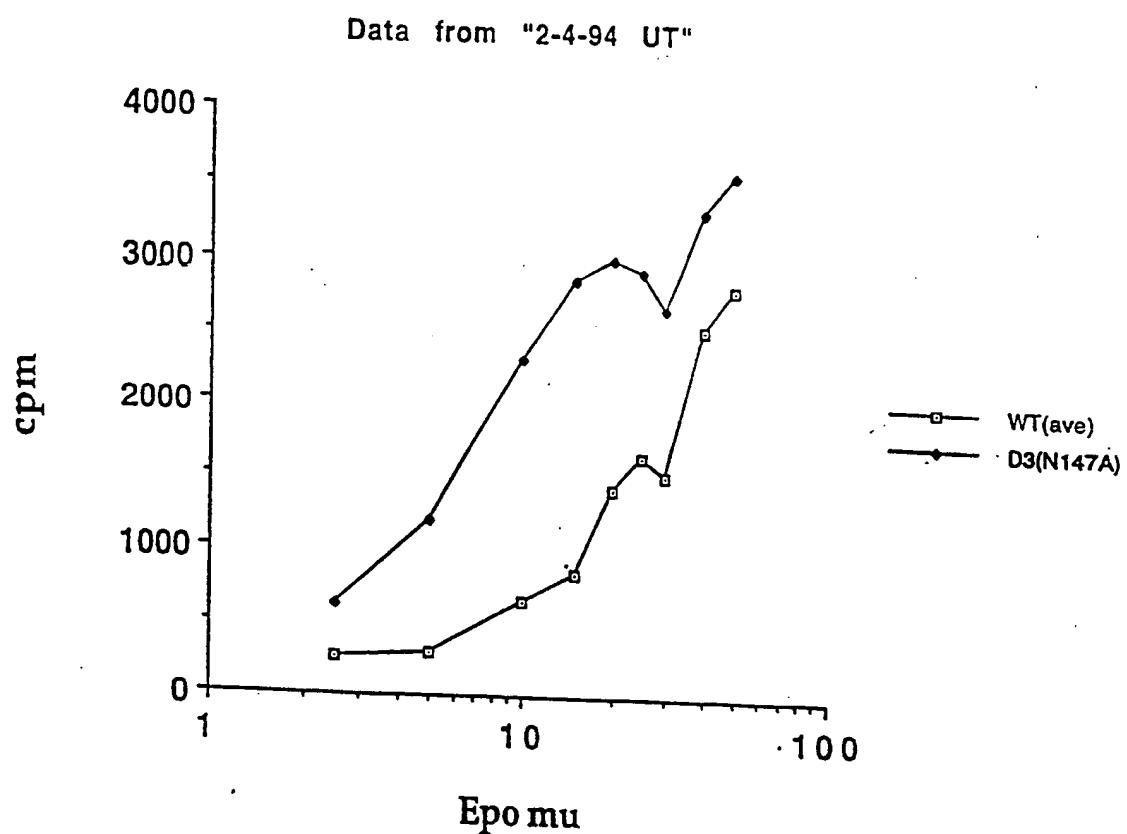


FIGURE 24

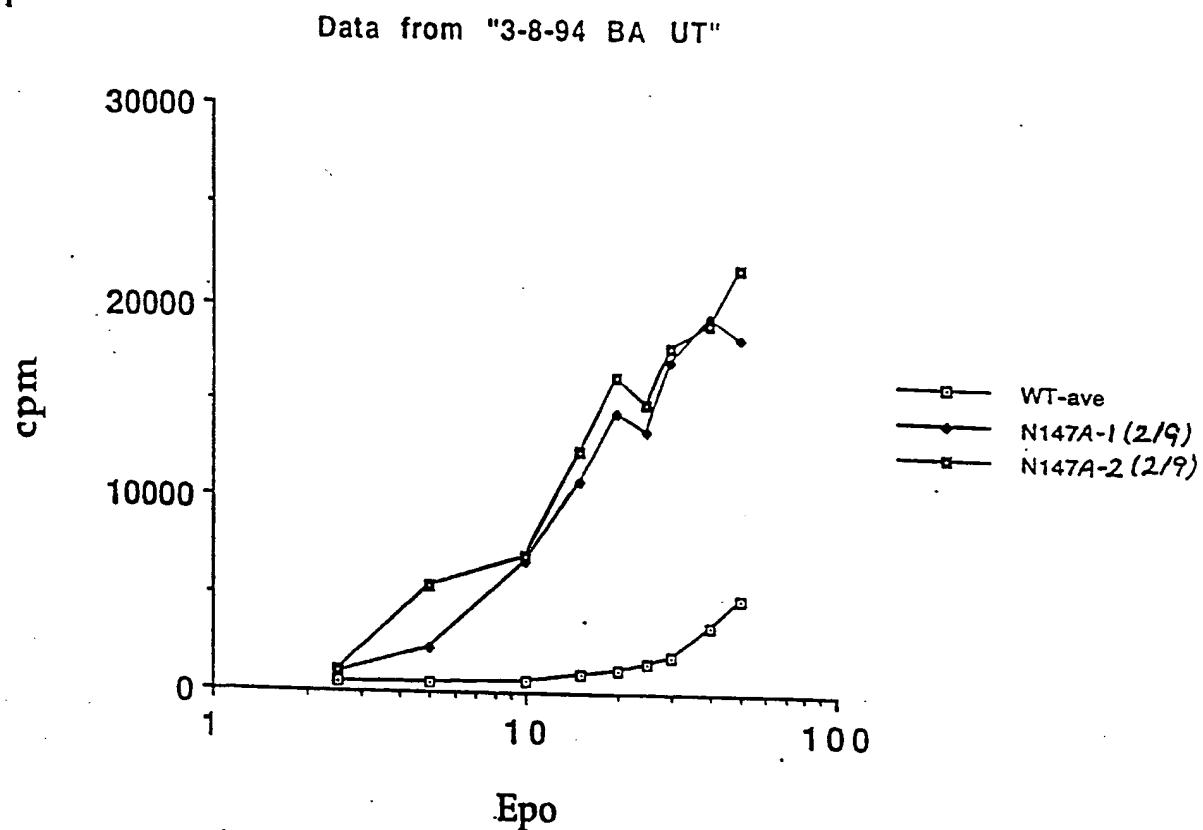


FIGURE 25

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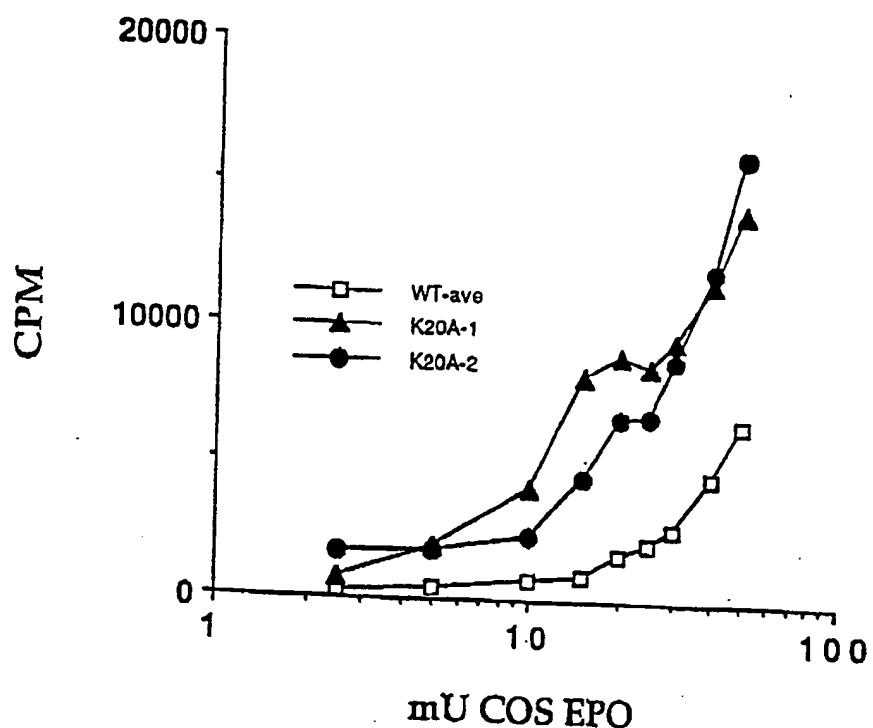


FIGURE 26

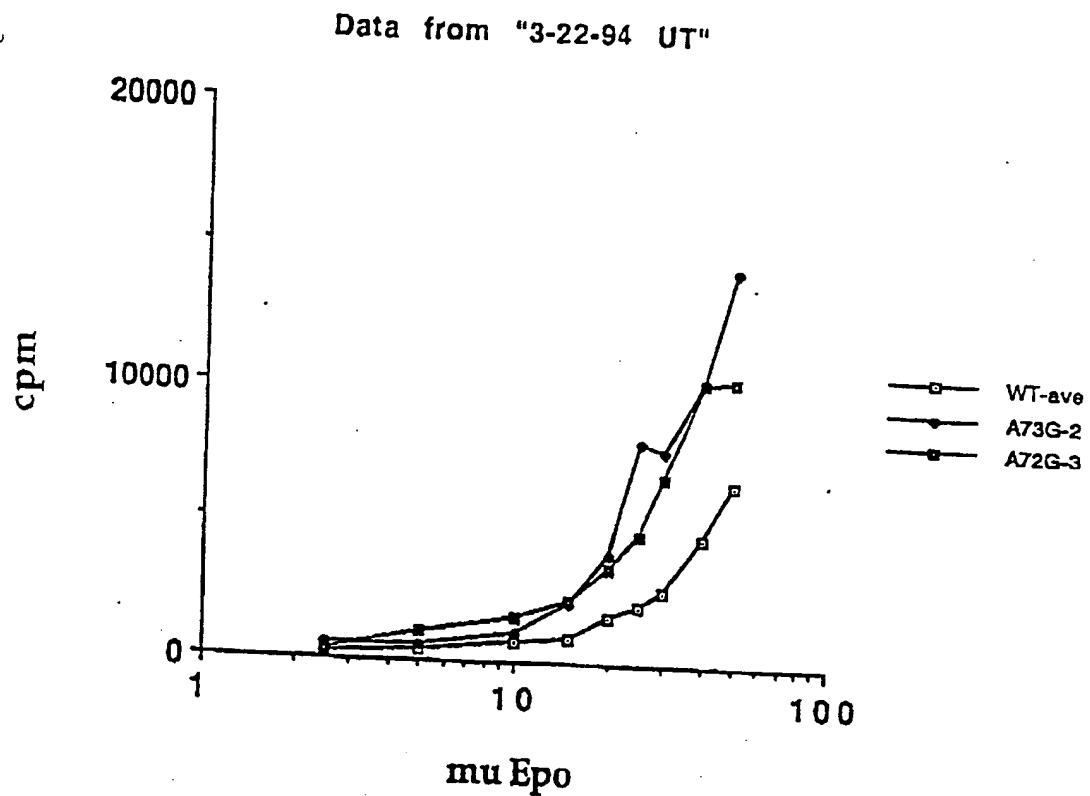
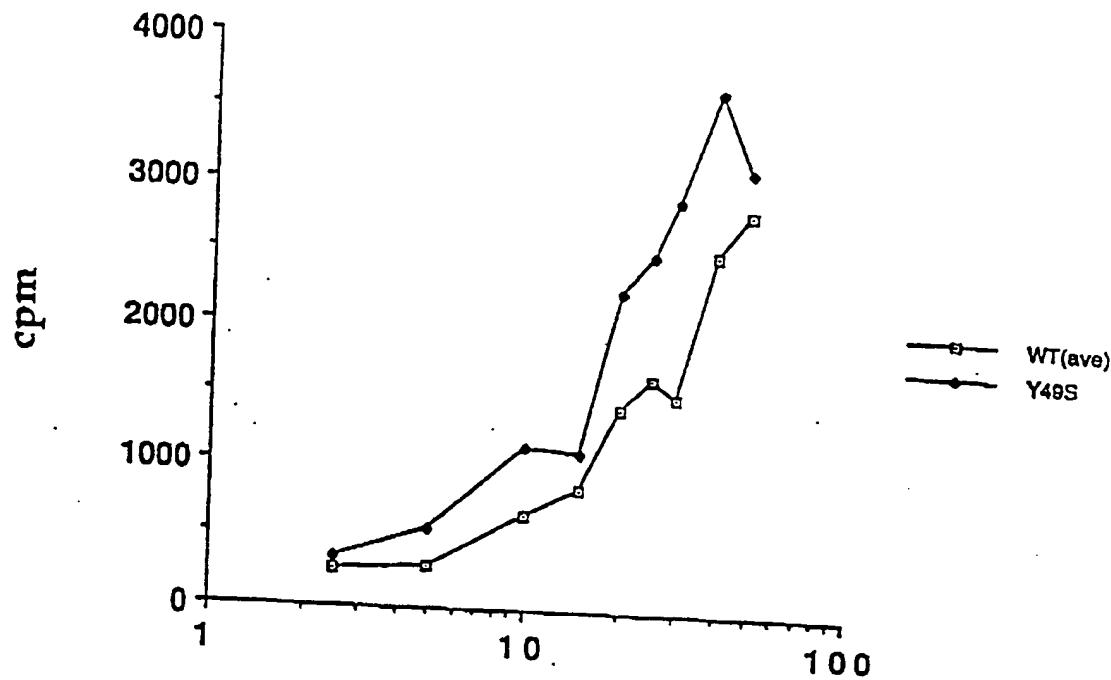


FIGURE 27

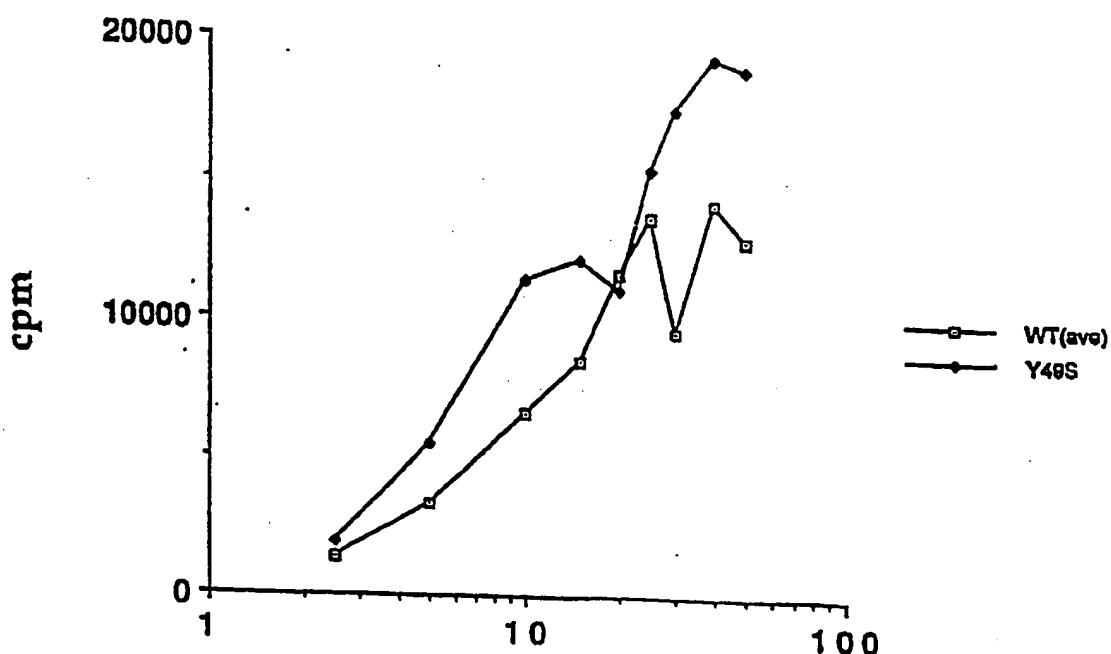
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Data from "2-4-94 UT"



Epo mu

Data from "2-4-94 HCD"



Epo mu

FIGURE 28

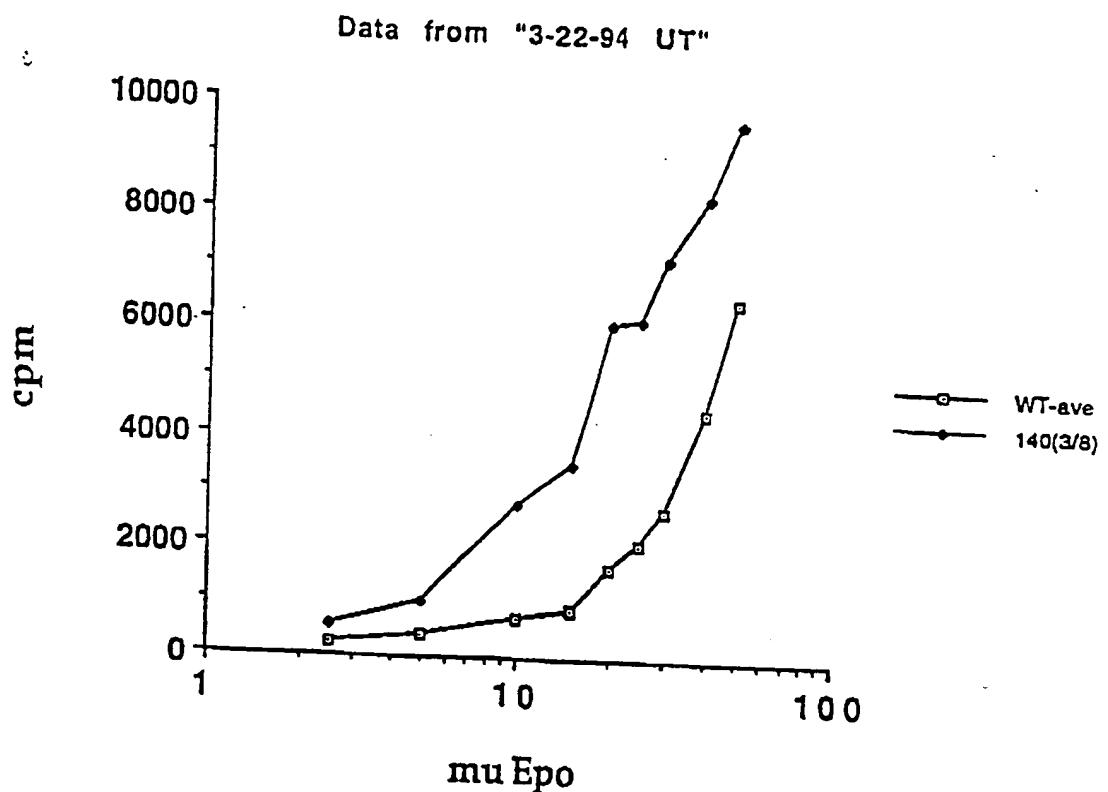
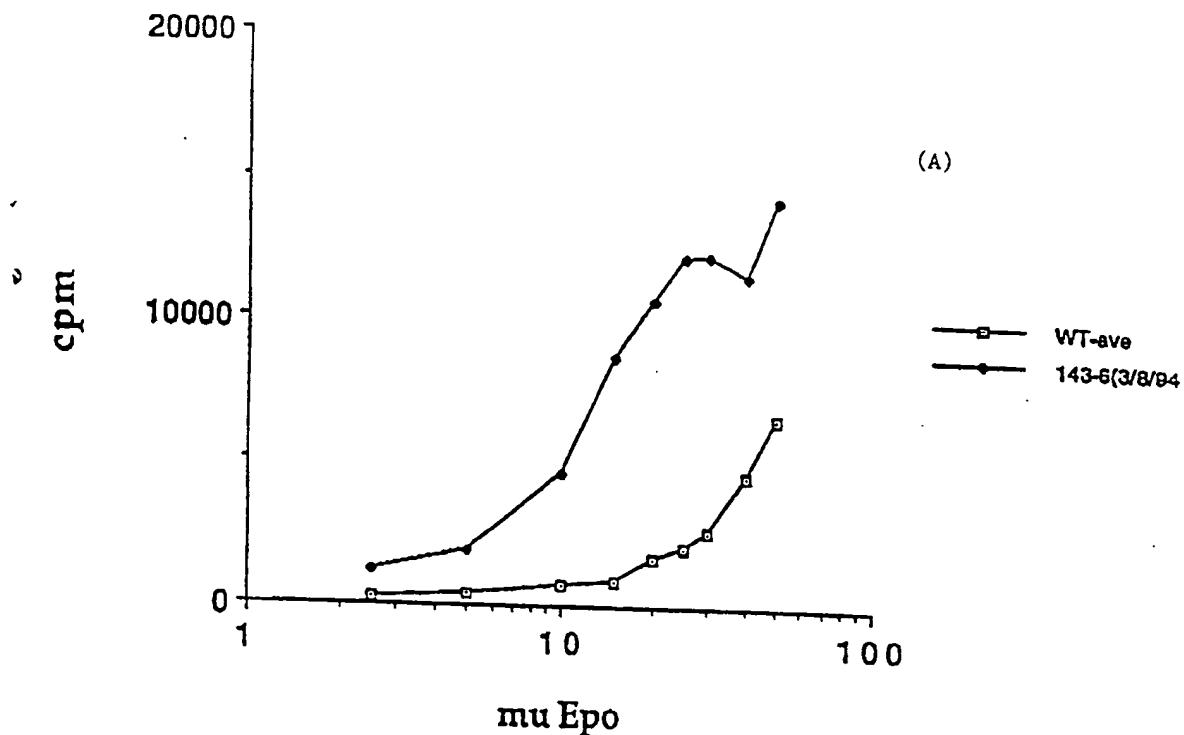


FIGURE 29

Data from "3-22-94 UT"  
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Data from "HCD BA 3-28-94"

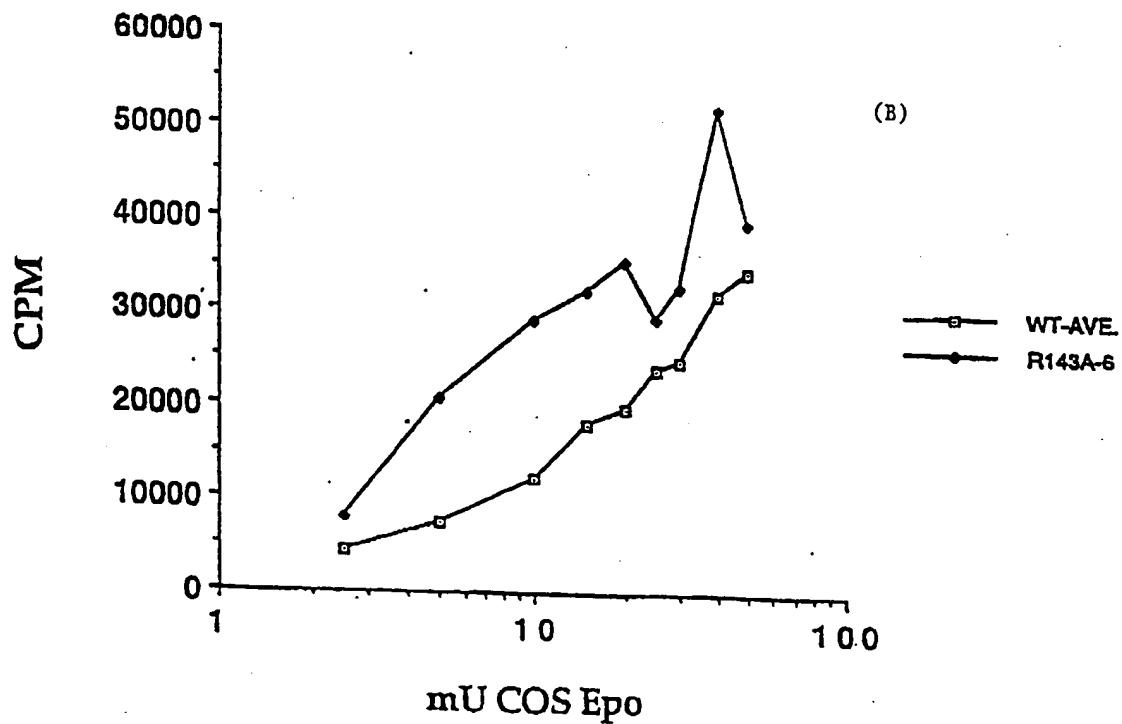


FIGURE 30

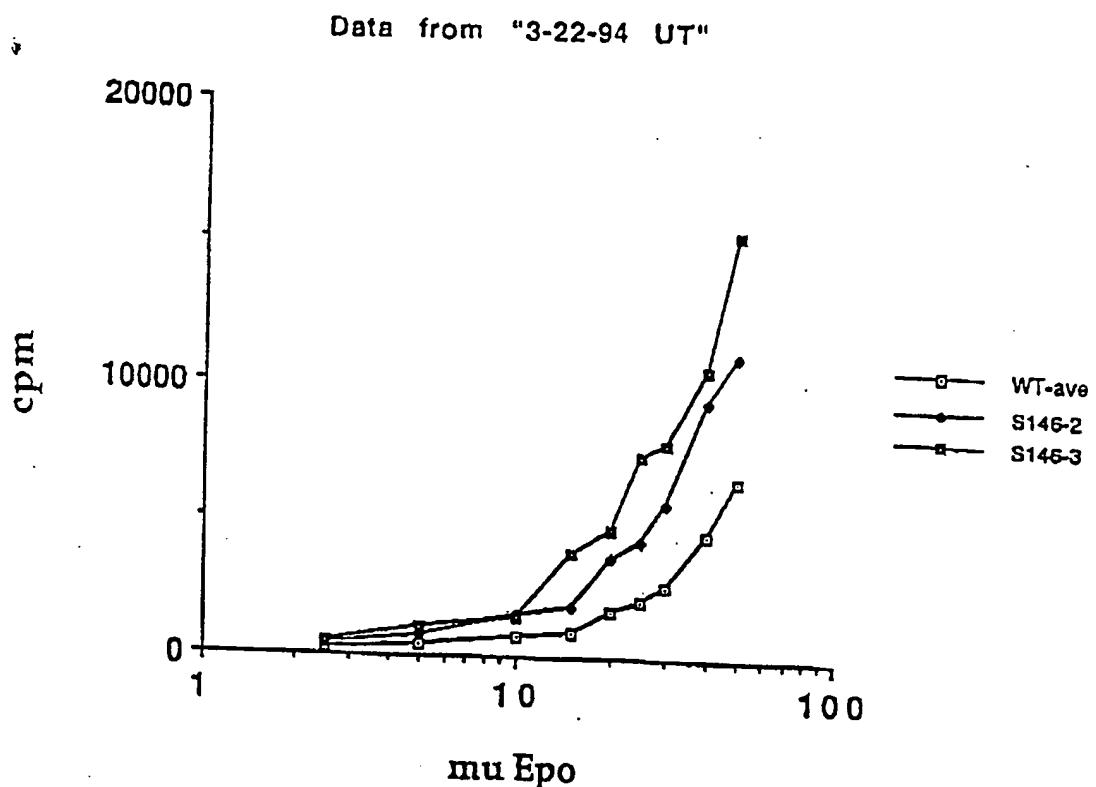


FIGURE 31

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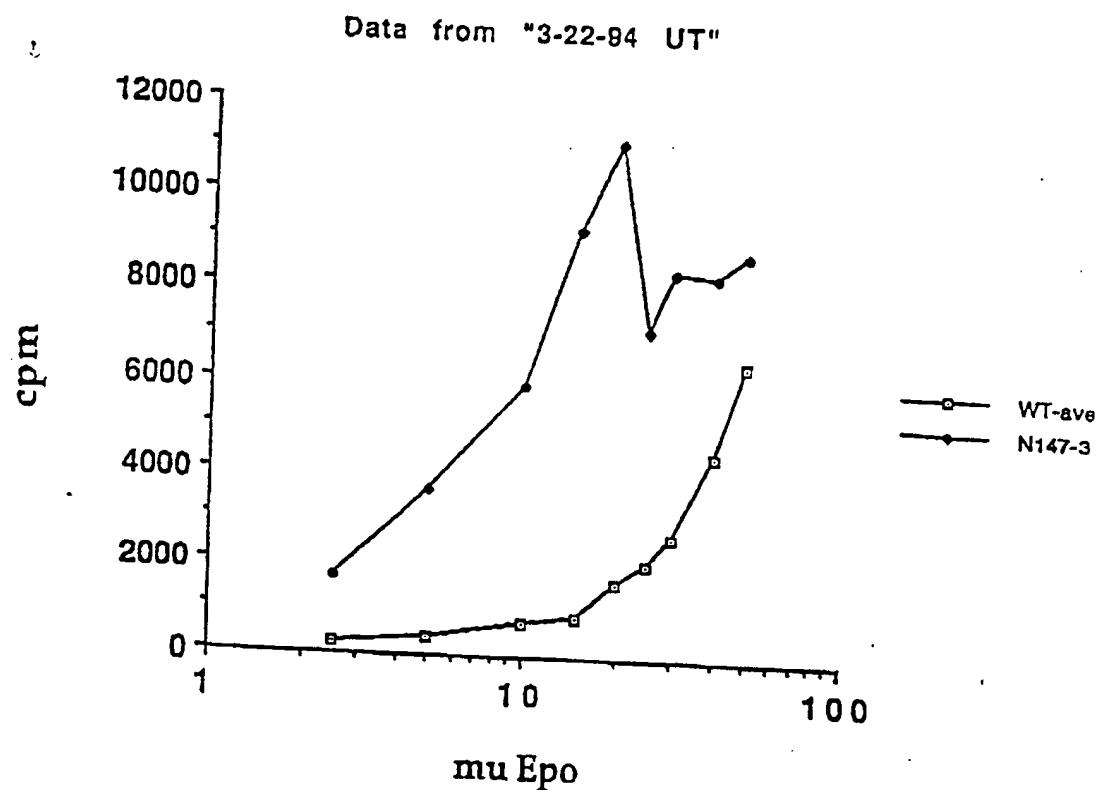


FIGURE 32

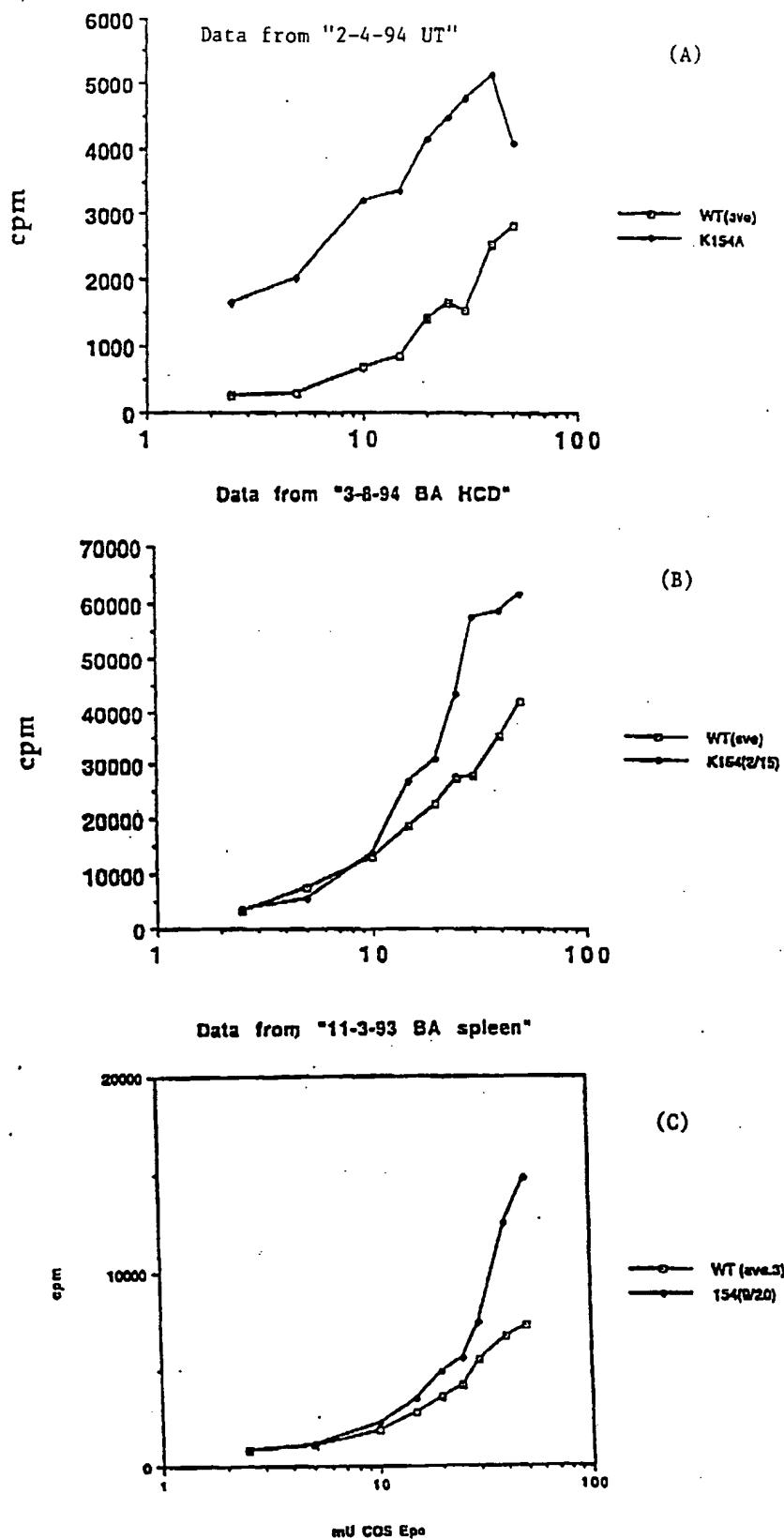


FIGURE 33

SPECIFIC BIOACTIVITY (% wt h EPO)				
Helix	Mutant	UT7	HCD	Spleen
A	S 9 A	148 ± 35 [3/3]	123 ± 13 [3/3]	-
A	R 10 A	91 [1/1]	123 [1/1]	-
A	E 13 A	111, 74 [2/2]	80, 99 [2/2]	-
A	R 14 A	58, 56 [2/2]	2, 30 [2/2]	18, 24 [2/2]
A	R 14 L	91 ± 8 [4/6]	86 ± 15 [4/6]	67, 111 [2/2]
A	R 14 E	11 ± 6 [3/5]	12 ± 1 [5/3]	17, 17 [2/2]
A	L 17 A	110 [1/1]	95 [1/1]	-
A	E 18 A	70 ± 17 [3/3]	100, 90 [2/2]	-
A	K 20 A	252 ± 81 [4/4]	103 ± 21 [4/4]	-
A	E 21 A	99, 69 [2/2]	50 [1/1]	-
A-B	C 29 Y / C 33 Y	75 [1/1]	42 [1/1]	-
A-B	K 45 A	110, 101 [2/2]	100, 100 [2/2]	-
A-B	F 48 S	146, 110 [2/2]	100, 61 [2/2]	-
A-B	Y 49 S	150 ± 7 [3/3]	146, 138 [2/2]	-
A-B	A 50 S	128, 92 [2/2]	140, 37 [2/2]	-
A-B	W 51 S	101, 78 [2/2]	89 ± 7 [3/3]	-
A-B	K 52 S	99, 90 [2/2]	99, 88 [2/2]	-
B	Q 59 A	156, 125 [1/2]	130, 134 [1/2]	-
B	E 62 A	101, 85 [2/2]	63, 78 [2/2]	82, 111 [2/2]
B	W 64 A	102, 112 [2/2]	87, 143 [2/2]	90, 107 [2/2]
B	Q 65 A	96 [1/1]	140 [1/1]	99 [1/1]
B	G 66 A	130 [1/1]	103 [1/1]	85 [1/1]
B	L 69 A	94 [1/1]	84 [1/1]	-
B	S 71 A	64 [1/1]	130 [1/1]	85 [1/1]
B	E 72 A	NS	-	-
B	A 73 G	242 ± 25 [2/3]	104 [1/1]	-
B	R 76 A	109 [1/1]	138 [1/1]	-

FIG.34

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Helix	Mutant	UT7	HCD	Spleen
C	Q 92 A	112 [1/1]	98, 95 [2/2]	113, 91 [2/2]
C	L 93 A	123, 126 [2/2]	127, 95 [2/2]	-
C	H 94 A	NS	-	-
C	D 96 A	NS	-	-
C	K 97 A	84 ± 9 [2/4]	77 ± 27 [2/3]	94 [1/1]
C	S 100 A	98, 85 [2/2]	131 [1/1]	104 [1/1]
C	G 101 A	166 ± 52 [3/3]	146 ± 26 [3/4]	73, 152 [2/2]
C	R 103 A	3 ± 3 [3/5]	7 ± 1 [2/3]	6 [1/1]
C	S 104 A	114 ± 28 [2/4]	35 ± 17 [2/3]	-
C	T 106 A	120 [1/1]	108 [1/1]	-
C-D	L 108 A	158 ± 41 [2/4]	48 ± 6 [2/4]	12, 52 [2/2]
D	D 136 A	108, 114 [2/2]	88 ± 20 [3/3]	-
D	R 139 A	171, 91 [2/2]	120, 109 [2/2]	90, 131 [1/2]
D	K 140 A	189 ± 30 [3/3]	110 ± 19 [3/3]	-
D	R 143 A	276 ± 85 [3/3]	150 ± 27 [3/3]	-
D	S 146 A	198, 269 [1/2]	58, 110 [1/2]	-
D	N 147 A	457 ± 185 [3/6]	115 ± 22 [5/6]	-
D	R 150 A	181 ± 94 [3/4]	92 ± 28 [3/4]	28, 64 [1/2]
D	G 151 A	11 ± 4 [2/3]	6 ± 2 [2/3]	-
D	K 152 A	89, 73 [1/2]	46, 20 [1/2]	16, 35 [1/2]
3'D	L 153 A	95 [1/1]	83 [1/1]	46 [1/2]
3'D	K 154 A	175 ± 60 [3/4]	185 ± 84 [3/5]	82, 157 [1/2]
3'D	L 155 A	95, 128 [2/2]	115 [1/1]	40, 98 [2/2]
3'D	Y 156 A	105 [1/1]	65 ± 12 [3/3]	-
3'D	T 157 A	86, 104 [2/2]	89, 91 [2/2]	102, 104 [2/2]
3'D	G 158 A	84, 109 [2/2]	94, 89 [2/2]	77, 139 [2/2]
3'D	E 159 A	89, 97 [2/2]	101 ± 10 [2/3]	33, 96 [2/2]

FIG.34A

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>5</sup> : <b>C07K 14/505</b>		A3	(11) International Publication Number: <b>WO 94/24160</b> (43) International Publication Date: <b>27 October 1994 (27.10.94)</b>
(21) International Application Number: <b>PCT/US94/04361</b>		(81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, GE, HU, JP, KG, KP, KR, KZ, LK, LU, LV, MD, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).	
(22) International Filing Date: <b>21 April 1994 (21.04.94)</b>			
(30) Priority Data: <b>08/049,802 21 April 1993 (21.04.93) US</b>			
(71) Applicant: <b>BRIGHAM AND WOMEN'S HOSPITAL [US/US]; 75 Francis Street, Boston, MA 02115 (US)</b>		Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(72) Inventors: <b>BOISSEL, Jean-Paul, R.; 15 Tanager Street, Arlington, MA 02174 (US). BUNN, H., Franklin; 184 Islington Road, Aubundale, MA 02166 (US). WEN, Danyi; 9 Sherman Road, Chestnut Hill, MA 02167 (US). SHOWERS, Mark, O.; 110 Longwood Avenue, Brookline, MA 02146 (US).</b>		(88) Date of publication of the international search report: <b>5 January 1995 (05.01.95)</b>	
(74) Agents: <b>CIMBALA, Michele, A. et al; Sterne, Kessler, Goldstein &amp; Fox, Suite 600, 1100 New York Avenue, N.W., Washington, DC 20005-3934 (US).</b>			
(54) Title: <b>ERYTHROPOIETIN MUTEINS WITH ENHANCED ACTIVITY</b>			
(57) Abstract			
<p>Novel modifications of erythropoietin (Epo) which improve the biological activity of the protein are provided. The modified Epo proteins (Epo muteins) may be used in the same manner as has been demonstrated for wild type Epo, except that relatively smaller doses are required due to the enhanced biological activity. Methods of using the Epo muteins for treatment of blood disorders are provided. A method of obtaining additional Epo muteins with enhanced biological activity is also provided.</p>			

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GA	Gabon				

**INTERNATIONAL SEARCH REPORT**

Int	ional Application No
PCT/US 94/04361	

**A. CLASSIFICATION OF SUBJECT MATTER**  
IPC 5 C07K14/505

According to International Patent Classification (IPC) or to both national classification and IPC

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Minimum documentation searched (classification system followed by classification symbols)  
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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 409 113 (BHERINGWERKE) 23 January 1991 cited in the application See the whole application, specifically Table 1 See the whole application	1-3,9, 13-25
Y	& AU,A,9 059 145 (BEHRINGWERKE) 19 July 1990 ---	4-8, 10-12
X	EP,A,0 148 605 (KIRIN-AMGEN) 17 July 1985 cited in the application See page 21, lines 19-33, page 91, line5 to page 92, line 2, claims 1-13, 43, 35, 55-57 & US,A,4 703 008 (KIRIN-AMGEN) 27 October 1987 ---	1,2, 13-20
	-/-	

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Date of the actual completion of the international search

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## INTERNATIONAL SEARCH REPORT

Int'l Application No  
PCT/US 94/04361

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